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Enhanced Activity And Stability Of Enzymes Associated With Delayed Fruit Ripening In Rhodococcus rhodochrous DAP 96253

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ENHANCED ACTIVITY AND STABILITY OF ENZYMES ASSOCIATED WITH DELAYED FRUIT RIPENING IN *Rhodococcus rhodochrous* DAP 96253

by

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Under the Direction of George E. Pierce

ABSTRACT

Rhodococcus has diverse metabolic capabilities, such as delaying ripening of certain climacteric fruit. Nitrile hydratase (NHase), amidase, 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase), cyanidase, and β-cyanoalanine synthase-like enzyme (βCAS-like) are possibly involved in fruit ripening. The activity of these enzymes in *Rhodococcus rhodochrous* DAP 96253 cells were induced with selected multiple inducers (i.e. cobalt and urea).

This research showed that the supplementation of selected sugars, i.e. trehalose and maltodextrin in growth media and storage buffers of *R. rhodochrous* DAP 96253 affected activity and stability of the enzymes mentioned above. Thermostability and osmostability of the five

enzymes in whole cells (plate grown and fermented) were evaluated in this study, i.e. βCAS-like was more stable than the other four enzymes in storage conditions.

Immobilized biocatalysts have practical advantages over the use of "free" whole cells. Immobilization of whole *rhodococcal* cells (plate grown and fermented) was employed, using techniques such as glutaraldehyde-polyethylenimine (GA-PEI) cross-linking, waxing and calcium-alginate entrapment. The GA-PEI immobilized catalysts were non-replicating and more stable in storage conditions than the catalysts produced by the other two methods. Wax or calcium-alginate immobilized catalysts (live catalysts) showed higher enzyme activity than the GA-PEI catalyst.

The effects of whole and immobilized catalysts were evaluated on delayed ripening of fruit. Both free whole cells and immobilized catalysts delayed the ripening of bananas and peaches. Delayed ripening experiments showed that the catalysts were effective in direct contact and not in contact with fruit. Moreover, both free whole cells and immobilized catalysts showed antifungal activity against *Aspergillus niger* and *Penicillium spp*.

Gas chromatography was performed to analyze volatile interactions between the biocatalysts and fruit. This analysis revealed that cyanide in an atmosphere with ethylene was utilized by the biocatalysts. There was also less volatile production by exposed fruit (bananas) than fruit unexposed to biocatalysts, either *rhodococcal* immobilized catalysts or live whole cells (plate grown and fermented).

INDEX WORDS: *Rhodococcus*, Fruit ripening, Biocatalyst, Growth media, Immobilization, Sugar, Stability

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1 INTRODUCTION

1.1 Genus of *Rhodococcus*

Rhodococcus is an aerobic, Gram-positive, mycolic acid-containing actinomycete that is widespread in terrestrial and aquatic environments (Finnerty, 1992; Warhurst and Fewson, 1994). Two species are pathogenic, *Rhodococcus fascians* which can induce leafy galls on host plants and causes fasciation in plants, and *Rhodococcus equi* which can cause respiratory infections of many animals (notably horses) and inhibits phagosome-lysosome fusion (Bell *et al*., 1998). The virulence of these two species is directly related to specific plasmids. The regions of specific DNA for identifying virulent plasmid-bearing strains of *R. equi* (a linear plasmid) and *R. fascians* (a circular plasmid) have been described in recent studies (Stange *et al*., 1996; Takai *et al*., 1995). The virulence plasmids of *R. equi* contain a highly variable region, which has a different GC-content from the plasmids in other *Rhodococcus* species. The major difference in genes between *R. fascians* and other *Rhodococcus* species is that genes controlling virulence on *R. fascians* plasmids contain three specific loci: *fas*, *att*, and *hyp*. Environmental *rhodococci* diverged from the pathogenic *R. equi* and *R. fascians*. Most environmental *rhodococci* are benign and non-pathogenic (Stange *et al*., 1996; Takai *et al*., 1995).

Rhodococci belong to the mycolata group of *Actinomycetes* (Table 1), with a high G+C content, and are important in bioremediation and industry due to their remarkable ability to catalyze a very wide range of compounds and their environmental robustness (Goodfellow *et al*., 1998; Van der Geize and Dijkhuizen, 2004; Warhurst and Fewson, 1994). The possession of a wide range of deoxygenases by *Rhodococcus* results in their ability to degrade aromatic compounds (Larkin *et al*., 2005). Moreover, because of their robustness, *Rhodococci* are well-suited

industrial biocatalysts. *Rhodococcus rhodochrous* DAP 96253 used in this study is non-sporulating, non-motile, and produced a red-orange non-diffusable pigment when grown on nutrient agar (Fig 1), and demonstrates the characteristics described for strains of *R. rhodochrous* in the Bergey's Manual of Systematic Bacteriology (Goodfellow, 1986). In addition, intergenic spacer (IGS) analysis confirms the designation of strain DAP 96253 as a *R. rhodochrous* strain (Pierce, unpublished).

Figure 1. *R. rhodochrous* DAP 96253 grown on nutrient agar plate

Table 1. Characteristics of wall chemotype IV general including *Segniliporus* [a], classified in the *Corynebacteriales* [b] (Goodfellow, 2012)

Characteristic	*Corynebacterium*	*Dietzia*	*Gordonia*	*Millisia*	*Mycobacterium*	*Nocardia*	*Rhodococcus*	*Segniliporus*	*Skermania*	*Smaragdicoccus*	*Tomitella*	*Tsukamurella*	*Williamsia*
Cell morphology	Pleomorphic rods, often club-shaped; commonly in angular and palisade arrangement	Short rods and cocci	Rods and cocci or moderately branching hyphae	Rudimentary right angled branching	Rods, occasionally branched filaments which fragment into rods and coccoid elements	Mycelium which fragments into rods and cocci	Rods to extensive substrate mycelium which fragments into irregular rods and cocci	Rods	Mycelium resembling a pine tree	Coccoid cells	Irregular rods that exhibit snapping division; cells turn to short coccoid rods after prolonged culture	Rods occur singly, in pairs, or in masses; coccobacillary forms occur	Thin irregular rods or cocci occur singly or in small clusters
Aerial hyphae	Absent	Absent	Absent	Present	Usually absent	Present	Absent	Absent	Present but not visible to the naked eye	Absent	Absent	Absent	Present
Time to growth of visible colonies (d)	1–2	1–3	1–3	1–3	2–40	1–5	1–3	3–4	9–21	7	nd	1–3	1–4
Acid-fastness	Sometimes weakly acid-fast	Not acid-fast	Partially acid-alcohol-fast	Acid-alcohol-fast	Strongly acid-fast	Partially acid fast	Partially acid-fast at some stage of the growth cycle	Acid–alcohol-fast	Not acid fast	nd	nd	Partially acid-alcohol-fast	nd
Strictly aerobic	–[d]	+	+	+	+	+	+	+	–	+	+	+	+
Fatty acid composition[e]	S, U[d]	S, U, T	S, U, T	S, U, T	S, U, T	S, U, T	S, U, T	S, U, T	S, U, T	S, U	S, U	S, U, T	S, U, T
Major menaquinone(s)[c]	MK-8(H_2)	MK-8(H_2)	MK-9(H_2)	MK-8(H_2)	MK-9(H_2)	MK-8(H_4,ω-cyel)	MK-8(H_2)	nd	MK-8(H_4,ωcyel)	SQA-8(H_4 ω cyel) and SQB (H_4 dicyel)	MK-9(H_2)	MK-9	MK-9(H_2)
Muramic acid type	Acetylated	Acetylated	Glycolated	Glycolated	Glycolated	Glycolated	Glycolated	nd	Glycolated	Glycolated	Glycolated	Glycolated	Glycolated
Mycolic acid pattern[f]	Single spot	Single spot	Single spot	Single spot	Multiple spots	Single spot	Single spot	Multiple spots	Single spot	nd	nd	Two spots	Single spot
Mycolic acids:													
Overall size (number of carbons)	22–38	34–38	46–77	44–52	60–90	46–64	30–54	nd	58–64	43–49	42–52	62–78	50–56
No. double bonds	0–2	0–1	1–6	nd	1–4	0–3	0–4	nd	2–6	nd	nd	1–7	nd
Fatty acids released on pyrolysis	8–18	nd	16–18	nd	22–26	12–18	12–16	nd	16–20	nd	nd	22–26	nd
Phosphatidylethanolamine present in polar lipid patterns	–[g]	+/–	+	+	+	+	+	nd	+	+	+	+	+
DNA G+C content (mol%)	51–67	66–73	63–69	64.7	57–73	63–72	63–73	68–72	67.5	63.7	69.5–71.6	68–78	64–65

[a]The wall chemotype of *Segniliparus* has yet to be established.

[b]Symbols: +, positive; –, negative; and nd, not determined. Data taken from Butler et al., (2005); Soddell et al., (2006); and Adachi et al. (2007).

[c]Abbreviations: S, straight chain; U, unsaturated; T, tuberculostearic acid (10-methyloctadecanoic acid).

[d]*Corynebacterium bovis*, *Corynebacterium minutissimum*, *Corynebacterium urealyticum* and *Corynebacterium vanpikii* contain tuberculostearic acid (Kämpfer et al., 1999; Lechevallier et al., 1977).

[e]Examples of abbreviations: MK-9(H_2), menaquinone with two of the nine isoprene units hydrogenated; SQA and SQB, smaradiquinones A and B.

[f]Number of mycolic acid spots produced from whole organism methanolysates (Minnikin et al., 1975, 1980). In mycobacterial mycolic acids, double bonds may be converted to cyclopropane rings; methyl branches and other oxygen functions may be present (Dobson et al., 1985).

[g]Present in *Corynebacterium bovis* and *Corynebacterium urealyticum* (Kämpfer et al., 1999).

1.2 Fruit Ripening

During ripening, dramatic changes in the fruit are controlled and regulated by a series of genes and metabolites. The following mechanisms: conversion of starch to sugar by amylase, metabolism of chlorophyll to anthocyanin by hydrolase, pectin degradation by pectinase, non-aromatic organic to aromatic by hydrolases, result in major changes of ripened fruit from sour to sweet, green to colorful, hard to soft, and odorless to aromatic (Agopian *et al.*, 2011; Kovács *et al.*, 2009). Fruits are characterized as climacteric or non-climacteric fruits based on their ripening mechanisms, with climacteric fruit experiencing a climacteric crisis which results in a great increase of ethylene production, which is accompanied by a respiratory peak and changes in the expression of a large number of genes (Pech *et al*., 2002).

Bananas and peaches are both representative climacteric fruit, which exhibit rapid ripening (Seymour, 1993). They are the most consumed fruits in the world, and bananas are usually harvested at unripe (mature green) stage and initiated to ripen (de-green) before marketing. Bananas are typically shipped "green" in a controlled atmosphere (CA) of oxygen and carbon dioxide to minimize ethylene biosynthesis. They are then forced in a CA of ethylene to initiate ripening (i.e. bananas picked green-ripe are gassed with a controlled atmosphere containing C_2H_4 to ripen to the yellow-ripe stage). Climacteric fruits undergo great changes including respiration, texture, color, ethylene and volatile production. The starch and sugar-contents also are important criteria in detecting stage of ripening (Barry and Giovannoni, 2007). The yellow color of ripened bananas is due to chlorophyll break down. It was reported that ethylene acted as the regulator of volatile compounds accumulation during bananas ripening (Yang *et al.*, 1984).

A large percentage of fruit and vegetable produce is lost worldwide postharvest, with much of the loss related to mechanical injury and microbial deterioration events that increase

during ripening. USDA estimates, an average that 40% of harvested fruits and vegetables are lost. As fruit turn soft during ripening, the potential for mechanical injury during transportation and storage increases. Reducing postharvest losses is gaining attention. Wax coating and fungicides are two major conventional methods, however, they are facing two major obstacles, public concern and proliferation of resistance (Tripathi and Dubey, 2009). In recent years, many cyclic propanes, such as 1-MCP and 3-MCP, which structures are similar to ethylene, have been used to improve shelf life and fruit quality. However, the potential disadvantages of using 1-MCP are that it might reduce the ethylene induced disease resistance and it does not reduce injuries from low temperatures or high CO_2 (Ku *et al.*, 1999). In this case, replacement or improvement of the conventional methods by natural products and microbial control is gaining considerable attention.

1.2.1 Ethylene

The plant hormone ethylene is important in root initiation and elongation, inhibition of seed germination, abscission, senescence, and ripening (Carbonell-Bejerano *et al*., 2011; Ma *et al*., 2003). Recent studies demonstrated that ethylene regulates many ripening-associated processes in climacteric fruit and vegetables with different ethylene sensitivity and production (Table 2), including the biosynthesis of aroma and flavor compounds such as esters and the increase of sugar content (Defilippi *et al*., 2005; Schaffer *et al*., 2007). Moreover, researchers have examined the relationship between ethylene production and perception by the plant and the accumulation of cell wall modifying proteins, which is closely related to disassembly of cell wall during fruit ripening and subsequently result in the ripening-associated fruit softening (Bennett and Labavitch, 2008). During different ripening stages of climacteric fruits, i.e. bananas, the ethylene production varies. In pre-climacteric period, ethylene level was low, followed by a sudden

burst as the ethylene peak mentioned above, which signals the beginning of ripening. After that, ethylene decreased to start ripening, and once arrived the post-harvest stage, the ethylene production increased respiration activity (Larotonda *et al.*, 2008). After this ethylene burst, ethylene biosynthesis decreased back to the pre-climacteric condition, resulting in a low ethylene level. Methods for delaying fruit ripening could be based on either blocking the ethylene signal transduction or lowering plant ethylene concentrations.

Table 2. Ethylene sensitivity/ production of fruits and vegetables (Thompson *et al.*, 2000)

Fresh fruits/ vegetables	Ethylene production	Ethylene sensitivity/perception
Apple	VH	H
Pear/ Apricot/ Avacodo/ Peach	H	H
Bananas	M	H
Cucumbers/ Tomato	L	H
Broccoli/ Cabbage/ Cauliflower/ Spinach	VL	H
Cut flowers - Roses/ Gladioli/ Chrysanthemums	VL	H
Flower Bulbs - Bulbs/ Corns/ Rhizomes/ Tubers	VL	H
Celery	VL	M
Potato	VL	M
Berries	L	L
Grapes	VL	L

VH: very high; H: high; M: medium; L: low; VL: very low

1.2.2 Ethylene Signal Transduction

Based upon gene analysis, Bleecker and Kende (2000) have proposed that a linear chain is generated in a signal transduction model (Fig 2; Bleecker and Kende, 2000). In higher plants, the perception starts with ethylene expression and interacts with the family of receptors associated with the endoplasmic reticulum membrane (ETR) (Giovannoni, 2004). Ethylene as the negative regulator affects the interaction of ETR family and constitutive triple response 1 (CTR1), which corresponds to a negative regulator of a membrane protein encoded by an ethylene-insensitive gene (EIN2). EIN2 positively regulates the downstream nuclear protein (EIN3) tran-

scription factor. The ethylene-response-factor (ERF1) gene promoter will be simulated by regulation of EIN3 transcription factors, and finally activating a set of responses to ethylene (Bleecker and Kende, 2000).

Models for blocking ethylene receptors could be established by either applying compounds that are agonistic or mimic/analogue ethylene. It was further reported that the ethylene blocking agents should control most ethylene responses in plants since the receptor was universal in plants. These agents were reviewed in recent papers, in which cyclooctenes (*trans* and *cis*) and heterocycles were major groups. Their inhibition effects were dependent upon their exposure time, concentration, structure and affinity to a metal (probably silver ion) that was provided in ethylene receptor (Burg and Burg, 1967). For blocking ripening of bananas and tomatoes, relatively high concentration of 2, 5-norbornadiene (NBD) is required, whereas a small amount of photolysis of diazocyclopentadiene (DACP) could work. Some reports mentioned that regained ethylene sensitivity after certain days was due to new receptors' synthesis. Ethylene competitors such as carbon monoxide, isocyanides and other olefins were reported. However, continuous treatment of some blocking agents, i.e. 2, 5 NBD, was required to delay ripening, while others were "permanent", i.e. strained olefins compounds, due to their dissociation time from the receptor (Sisler and Serek, 1999).

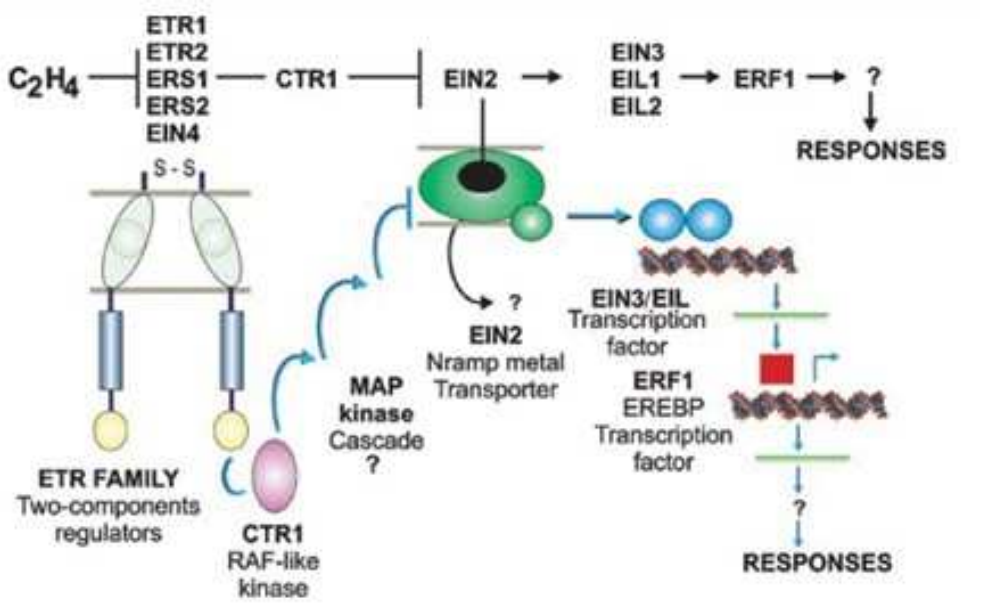


Figure 2. Ethylene signal transduction pathway (Bleecker and Kende, 2000)

1.2.3 Ethylene Synthesis

Compared to the partial understanding of ethylene perception and signal transduction pathway, there is a well-established model for ethylene biosynthesis pathway in higher plants. S-adenosyl-L-methionine (SAM) is synthesized from methionine by SAM synthase, and converted to 1-aminocyclopropane-1-carboxylate (ACC) by ACC synthase. ACC is then subsequently converted to ethylene by ACC oxidase; hydrogen cyanide (HCN) and carbon dioxide (CO_2) will be subsequently catalyzed by HCN metabolic enzymes (Fig 3; Adam and Yang, 1979). Recent study on genes encoding these enzymes has demonstrated that they were differentially expressed during development, and the difference was also shown in response to internal or external stimuli (Fluhr and Matoo, 1996).

Models for lowering plant ethylene concentrations could be established by either inhibiting ethylene synthesis or increasing the rate of ethylene degradation. All plants in nature harbor a diverse community of bacteria. The relationship between plants and their environmental bacteria has been described by many researchers. Many plant growth-promoting factors such as anti-

biotics, indole-3-acetic acid, ACC deaminase, and siderophores are known to be produced during the stationary phase of bacteria or by bacteria under some environmental stress (Hontzeas *et al.*, 2004). Based on this information, it is reasonable to predict that bacteria possess enzymes involved in ethylene metabolism, and be potentially used in affecting fruit ripening.

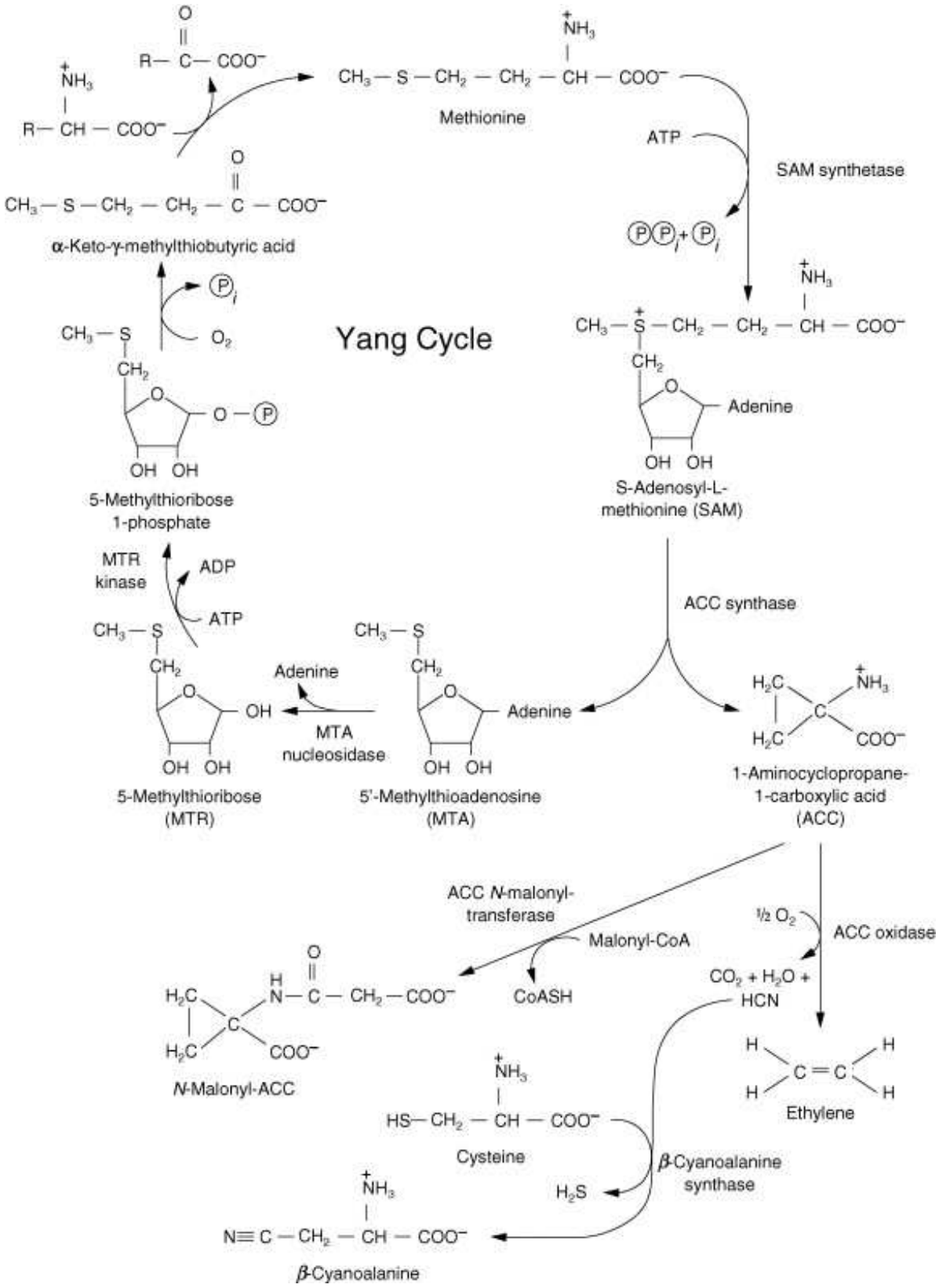


Figure 3. The Yang Cycle and formation of ethylene (Yang and Hoffman, 1984)

1.3 Enzymes Related to Fruit Ripening

1.3.1 ACC Deaminase, Cyanidase and β-cyanoalanine Synthase (βCAS)

Hoffman and Yang (1980) noted that there was a relationship between the changes in ACC content and ripening of fruits and vegetables via ethylene biosynthesis. ACC deaminase is a common enzyme found in many soil microorganisms, including both bacteria and fungi, and also in some plants. ACC deaminase is a pyridoxal 5'-phosphate dependent enzyme, which belongs to the hydrolase family of enzymes. ACC deaminase acts on carbon-nitrogen bonds other than peptide bonds, resulting in the cleavage of the cyclopropane ring of ACC (the immediate precursor of plant hormone ethylene), resulting in the formation of α-ketobutyric acid and ammonia as the products (Hontzeas *et al.*, 2004; Ose *et al.*, 2003; Todorovic and Glick, 2008).

A model for the lowering of plant ethylene concentrations by expressing ACC deaminase in the desired plant will lower the level of ACC, thereby reducing the level of ethylene in the desired plant tissue which in turn results in fruit ripening delay (Adams and Yang, 1979; Glick *et al*., 1998). Meanwhile, ACC oxide has been reported to be distributed throughout the pericarp of fruit during ripening, and cobalt could inhibit ethylene production and ripening as an ACC oxidase inhibitor. It also has been reported that the infiltration of alpha-aminooxyacetic acid (AOA) or aminoethoxyvinyl glycine (AVG) could inhibit the ethylene production and substantially color development of saskatoon fruit (Rogiers *et al.*, 1998). Since ACC deaminase does not bind its substrate ACC with high affinity, in order to compete with ACC oxidase, ACC deaminase must be present in much greater amounts (Hao *et al.*, 2011). It is reasonable to predict that bacteria with very high levels of ACC deaminase could have the potential to lower plant ethylene levels, and thereby help to delay the ripening process of fruit and extend the harvesting season and shelf life for these goods.

It is known that cyanide occurs naturally in certain plants and microorganisms (Vetter, 2000). HCN is a co-product in ethylene synthesis (Fig 3; Adam and Yang, 1979). The enzymes that participate in the cyanide metabolism could have an effect on fruit ripening via influencing the ethylene biosynthesis. Certain plants and surrounding microorganisms contain two major enzymes that catalyze cyanide: cyanidase and beta-cyano-alanine synthase (βCAS) (Fusao *et al*., 1992). Cyanidase, a member of the hydrolase family, hydrolyzes cyanide to form ammonia and formic acid (Ketterer and Keusgen, 2010). Detoxification of cyanide also is attributed to βCAS through the conversion of cyanide to β-cyanoalanine, which can further affect the amino acid pool (Fig 4; Urbanska *et al*., 2002). In some bacteria and higher plants, (i.e. spinach), βCAS functions in detoxifying HCN to asparagine (Wurtele *et al*., 1985). βCAS belongs to β-substituted alanine synthase family (Ogunlabi and Agboola, 2007; Siegien and Bogatek, 2006; Watanabe *et al*., 2008). After response to ethylene, it was reported that βCAS participated in supplying asparagine; this resulted in an increased amino acid pool in cocklebur seeds, which was observed during the pre-germination period (Maruyama *et al*., 1997). Microorganisms could adapt to the toxic effects of the cyanide released by the plants by producing βCAS-like enzymes.

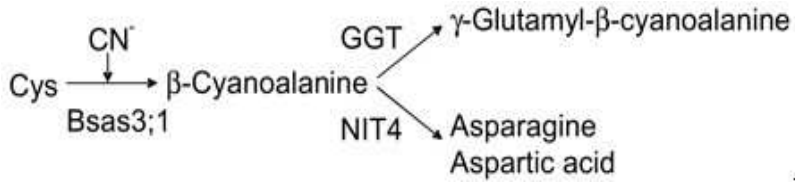


Figure 4. Metabolism of cyanide by βCAS (Watanabe *et al.*, 2008)

1.3.2 Nitrile Hydratase (NHase) and Amidase

A number of nitriles and nitrile-containing compounds have been shown to take part in metabolic cycle of higher plants and soil microorganisms, including some growth hormones for plant, natural metabolites, 3-Indoleacetonitrile and its derivatives, and cyanoglycosides (Conn,

1980). NHase produced by *Rhodococcus* and other microorganisms could play a key role in the process of conversion of the many chemically diverse nitriles to their corresponding amides. These amides can be further catalyzed into corresponding acids and ammonia production through amidase catalysis (Fig 5; Bunch, 1998; Ingvorsen *et al*., 1988).

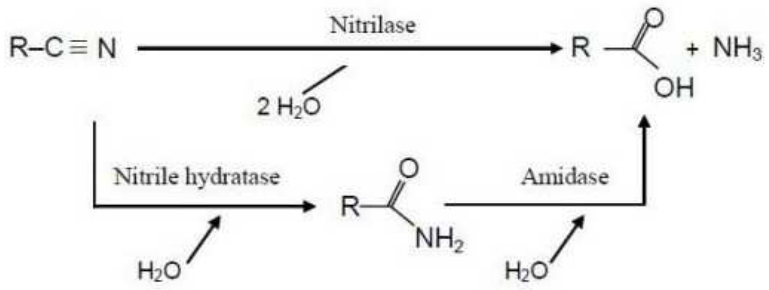


Figure 5. Degradation of nitrile (Rao *et al.*, 2010)

NHase has gained increasing attention since its wide application in industrial amide production and environmental bioremediation (Wyatt and Knowles, 1995; Yamada and Kobayashi, 1996). This enzyme is a soluble metalloenzyme. Many researchers have conducted research to establish the role of either non-heme iron or non-corrin cobalt on the active site of NHase (Mascharak, 2002). NHase is composed of α subunits and ß subunits, forming either αβ heterodimeric or $(\alpha\beta)_2$ tetrameric structures (i.e. $[\alpha\beta]_2$). It was shown that the iron-containing NHase from *Rhodococcus sp.* R312 contained one iron atom per αβ unit (Huang *et al*., 1997). The cobalt-containing NHase found in some species belongs to the small enzyme family that uses cobalt as a cofactor. In general the non-corrin NHase are more robust and have wider substrate specificity than the iron-containing NHase (Bishop and Sewell, 2006; Miyanaga *et al.*, 2004). The metal binding domain, three cysteine residues and one serine residue that co-ordinate metal, is in the α subunit, while two arginine residues are in the β unit, and it is further reported that these sites are highly conserved (Fig 6; Brennam *et al*., 1996; Mascharak, 2002). The iron-containing NHase shows photoreactivity, binds a nitric oxide molecule, and hydrates small ali-

phatic nitriles, whereas cobalt-containing NHase prefers to hydrating aromatic nitriles, which may be caused by the difference of their substrate binding pocket (Miyanaga *et al*., 2003). It was suggested by Yamada and Kobayashi (1996) that a high-molecular-mass NHase, which is used for industrial acrylamide production, could be produced by *Rhodococcus rhodochrous* when induced by substrates. However, when induced by urea, *Rhodococcus rhodochrous* could produce a low-molecular-mass NHase.

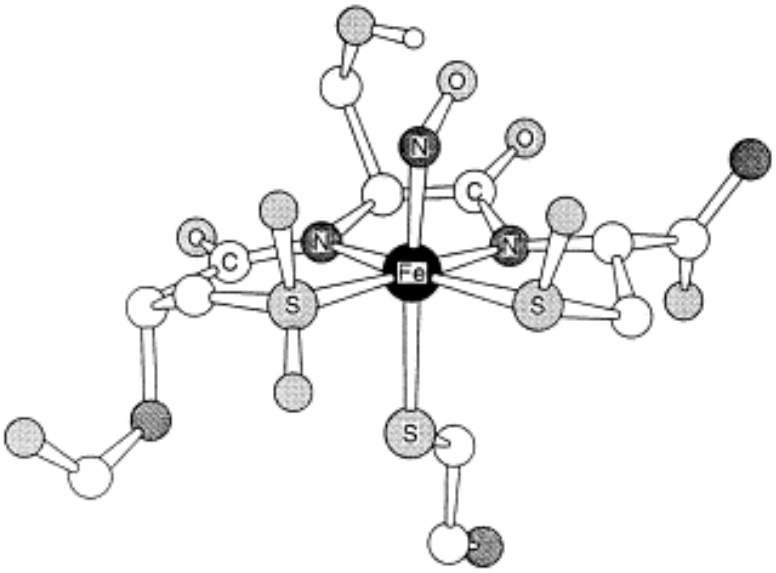


Figure 6. Structure of the nitric oxide-bound iron site of *Rhodococcus sp.* N-771 NHase (Huang *et al.*, 1997)

Amidase participates in several metabolic pathways. The exact physiological role of amidase is not fully understood. In some species, amidase along with NHase are involved in the use of nitriles as nitrogen sources (Cowan *et al*., 1998; Novo *et al*., 2002). Amidase has four families distinguished based on the cleavage of C–N bonds by the enzymes. These include hydrolase, urease, amidase signature and nitrilase (Novo *et al*., 1995; Rigden *et al*., 2003). Recent research showed that an aliphatic amidase from a *Rhodococcus* species was related to nitrilase and the cyanide hydratase family (Pertsovich *et al*., 2005). The active site of amidase from *R. rhodochrous* J1 resides with amino acid residues Asp191 and Ser195 (Novo *et al.*, 1995).

The relationship between specific amidase and fruit ripening was reported, for example, PNGase (peptide N4 (N-acetyl-glucosaminyl) asparagine amidase) and ENGase (endo N-acetyl-D-glucosaminidase) are involved in degradation of tomatoes by releasing of N-glycans from N-glycoproteins (Faugeron *et al*., 2006). Hence, the amidase produced by *Rhodococcus* could have an effect on fruit ripening.

Many nitrile-containing compounds are essential components in plant growth via germination and phototropism. There is a possible model for the ethylene metabolic pathway model that relates to NHase and amidase as follows. Ethylene can be oxidized by oxygenase (AMO) to ethylene oxide (Hommes *et al.*, 1998). This product can react with HCN or cyanide ions and form the corresponding cyanohydrin. In the case of ethylene oxide, the cyanohydrins created from ethylene oxide will spontaneously decompose to acrylonitrile. Since cyanohydrins are putative substrates for NHase, they could be degraded by the two step reactions: first converted to the corresponding amides by NHase, and then to acid salts by amidase (Gerasimova *et al*., 2004).

1.4 Induction of the Enzymes Involved in Fruit Ripening in *Rhodococcus*

As a result of their remarkable production of diverse enzymes, *Rhodococcus* species are known to play a critical role in biodegradation of volatile organic compounds (Bell *et al.*, 1998). During ripening, climacteric fruit will have a large increase of volatile aromatic compounds. Fruit ripening is related to ethylene production. Therefore, ethylene levels in plants are affected by the ratio of nitrate to nitrogen in the environment (Ligero *et al*., 1999). Recent research described that the growth and yield of maize were improved by the ACC deaminase in *Pseudomonas spp.* in the presence of nitrogenous fertilizer (Shaharoona *et al*., 2006). Urea, ammonium nitrate (NH_4NO_3) and potassium nitrate (KNO_3) fertilizers were used as the nitrogen source. Urea was reported to be an inducer of high-molecular-mass NHase. Cyclohexanecarboxamide in

the presence of cobalt ions could induce low-molecular-mass NHase in *Rhodococcus* (Komeda *et al.*, 1999). It is possible that ACC deaminase and other ethylene related enzymes may also be induced in *Rhodococcus rhodochrous* DAP 96253 by increasing nitrogen level. Researchers have reported that the ACC deaminase containing bacteria showed resistance to heavy metals, such as nickel, lead, zinc, cadmium and chromate ions, which was attributed to a lower level of stress ethylene which was in plant induced by these toxic metals (Arshad *et al*., 2007). Since ethylene also acts as a stress signal in plants, some ethylene related enzymes could be induced by stress conditions. The change of cell envelope and growth media by different carbohydrates could influence the enzymes activities inside.

1.5 Sugars

1.5.1 Sugars and Mycolic Acids

Mycolic acids (MA) are large 2-alkyl 3-hydroxy branched-chain fatty acids found in Mycolata group. MA in *Rhodococcus* and *Mycobacteria* attach to an arabinogalactan cell wall polysaccharide. With the arabinogalactan also linked to peptidoglycan, a peptidoglycan–arabinogalactan–mycolic acid complex is assembled in the Mycolata (Chun *et al*., 1996; Sutcliffe, 1998). The extremely long chained mycolic acids interacts with the outside carbon chains of the surface mycoloyl glycolipids, such as trehalose 6,6′-dimycolate and glucose monomycolate (Enomot *et al*., 2005). Some anti-tuberculosis drugs were designed by inhibiting biosynthesis of mycolic acids, which indicated the essential of mycolic acids for survival of mycobacteria (Banerjee *et al.*, 1994).

MA, which may make up 30% of the *Rhodococcus rhodochrous* cell wall skeleton by weight, also could be partially free as either trehalose dimycolatesor monomycolic lipids (Fig 7; Lang and Philp, 1998; Nishiuchi *et al*., 2000; Sutcliffe, 1998). MA, as the outer lipid permeabil-

ity barrier in this complex, could enhance the resistance of cells to chemical injury and dehydration, and reduce the uptake of hydrophilic substrates as well (Barry *et al*., 1998).

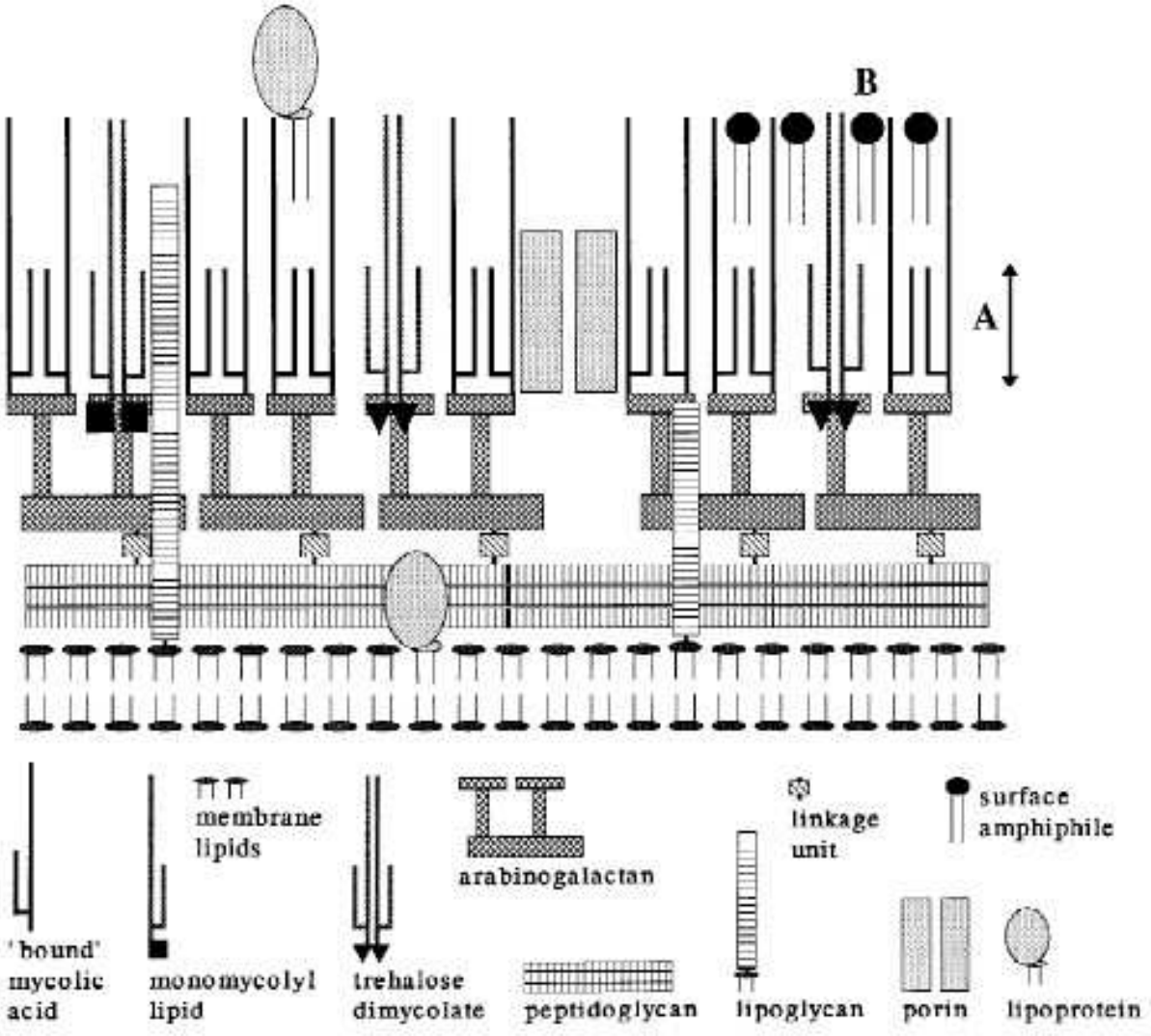


Figure 7. Cell envelope schematic in *Rhodococcus* (modified from Sutcliffe, 1998)

A change in temperature resulted change in a cell wall lipid composition (Baba *et al.*, 1989). Recently study showed that glucose monomycolate was produced in abundance at reduced temperature (Enomoto *et al.*, 2005). The fluidity of the cell changes with temperature. MAs produced at low temperature are different from those produced at higher temperature. MAs act as the barrier of the cell and are involved in membrane fluidity and impermeability. In this

case, changing in mycolic acids biosynthesis may influence cell stability. Study with *Bacterionema matruchotii* cells detected that the localization of mycolic acid biosynthetic activities was only in the cell wall fraction, not in the inner membrane. Various sugars, such as glucose and maltose, were absolutely required for mycolic acids biosynthesis. Addition of trehalose could result in a shift from glucose mycolate to trehalose monomycolate (Shimakata *et al.*, 1986). In a study with *Mycobacterium tuberculosis*, trehalose and mycolic acids were essential for growth (Gebhardt *et al.*, 2007).

A recent study showed that the presence of sugars in growth media caused changes of mycolic acid, and further affected the production and stability of nitrile hydratase in *Rhodococcus* (Tucker *et al.*, 2012). Based on the above information, the five enzymes produced by *Rhodococcus rhodochrous* DAP 96253 also may be affected by the change of mycolic acids according to various sugars in growth media.

1.5.2 Sugars and Stabilization of Enzymes

The stability of a biocatalyst is a major concern in any biocatalyst-related process. The five enzymes: NHase, amidase, cyanidase, ACC deaminase, and βCAS-like in this study, attracted increasing attention in recent years as a result of their critical roles in organic synthesis, fruit ripening and plant growth regulation. The stability of these enzymes produced by *R. rhodochrous* DAP 96253 varied. Stablity can great affect the application of the bacteria to commercial processes. To retain the activity of enzyme in whole cells, the complexities, efficiencies, and costs of the stabilization techniques need also be considered. Three common methods that could be applied to enhance enzyme stability include: modifying the storage conditions and storage buffers, and enzyme immobilization.

Changes in temperature, pH, and water content could influence the fatty acids (FAs) in the *Rhodococcus* cell wall. FAs confer cell stability: membrane fluidity and impermeability (Beney and Gervais, 2001; Ristau and Wagner, 1983; Whyte *et al*., 1999). Recent studies determined that the change of hydrocarbon growth substrates has a significant effect on the MA content in *Rhodococcus* and the cell wall structure (Sokolovská *et al*., 2003; Wick *et al*., 2002). Different storage carbohydrate buffers can influence the enzyme stability inside by affecting stability and permeability of cell envelops under different storage conditions, such as low temperature and pH.

Rhodococcus has become an important genus for acrylamide production and many other applications in industry and academia. For high-throughput applications or long term storage, the enzyme activities of *Rhodococcus* need to be preserved at various storage temperatures under dehydrated condition outside laboratory environments. Therefore, the osmostability of enzymes in *Rhodococcus* whole cells was examined in this research.

Sugars (e.g. Glucose, sucrose, and trehalose) are major constitutive protective substances. Many researchers have demonstrated that sugar supplements improved the stability of dehydrated cells and post-dehydration cells, and during desiccation. Increasing amounts of soluble sugar was shown contribute to membrane stabilization (Wu *et al.*, 2012). There are two possible mechanisms by which sugars can protect the dehydrated cells: (1) the formation of an intracellular glass-state during desiccation or freezing; (2) the formation of hydrogen bonds and substitution for water in maintaining hydrophilic structures (Crowe *et al.*, 1988; Leopold and Vertucci, 1986).

1.6 Immobilization

Immobilized enzymes are utilized more often in industry than free enzymes due to their specificity, stability, and reusability. It is generally known that the free enzymes have poor storage stability in solution, and recovery of soluble enzymes for re-use is difficult. Biological macromolecules, such as calcium alginate and glutaraldehyde, are frequently used in immobilization. Moreover, immobilization of the enzymes in calcium alginate, glutaraldehyde or wax could result in varied enzyme activity and increased stability. Among the different methods, cross-linking and entrapment have attracted significant attention, for its simplicity and direct immobilization of enzymes (Scheller *et al*., 1992).

The use of edible coatings such as cellulose, alginate, and wax represent common methods used to help prolong the preservation of fruits or vegetables by reducing respiration losses (Tapia *et al*., 2008). Many acid polysaccharides can be used to form stable films. Alginate, for instance, forms the polysaccharide-based coating by cross-linking with calcium ions (Tapia *et al*., 2008).

Glutaraldehyde immobilization is rooted deeply in biotechnology and is based on cross-linking the enzyme to a support matrix. Its mechanism is rapid protein adsorption by ionic exchange, followed by the covalent attachment of proteins to preexisting solids (Balcao *et al*., 2001; Mieglo *et al*., 2003).

In order to prepare biodegradable and edible film coatings on fruits and vegetables, waxes are widely used. As a barrier to gas and moisture, they are considered to be most efficient (Sreenivas *et al*., 2011). Animal, vegetable, mineral oil and synthetic waxes are all applied on fruit, for instance, shellac and carnauba for apples, mineral oil for tomatoes, and various waxes

for cucumbers (Hoa *et al*., 2002). Recent studies have already shown successful immobilization of enzymes with wax that maintain most of the enzyme activity (Pithawala *et al*., 2010).

2 MATERIALS AND METHODS

2.1 Microorganisms

Four microorganisms: *Rhodococcus rhodochrous* DAP 96253 (ATCC 55899), *Rhodococcus rhodochrous* DAP 96622 (ATCC 55898), *Rhodococcus erythropolis* (ATCC 47072), *Pseudomonas aeruginosa* GSU3 were obtained from American Type Culture Collection (ATCC, VIENNA, VA). *Rhodococcus rhodochrous* DAP 96253 was chosen for induction and stabilization research.

All strains were revived and maintained according to the ATCC manual. Cell culture was started from a glycerol stock (stored at -80˚C) by transferring 1 ml of the glycerol stock into 75 ml nutrient broth (Difco, Sparks, MD). The culture was incubated at 30°C while shaking at 150 rpm for two days. Nutrient agar (Difco, Sparks MD) plates were inoculated from the revived nutrient broth culture and grown for three days at 30°C. Cells scraped from these plates were used as an inoculum for yeast extract malt extract (YEMEA) plates (Dietz and Thayer, 1980) with various inducers. After seven days grown in the solidified media at 30°C, cells were harvested for further use.

2.1.1 Comparison of Enzyme Production among the Strains

NHase, amidase, cyanidase, ACC deaminase, and βCAS-like enzyme levels of cells (*R. rhodochrous* DAP 96253, *R. rhodochrous* DAP 96622, and *Pseudomonas aeruginosa* were grown on YEMEA with cobalt and urea, while *R. erythropolis* 47072 were grown on Nutrient Agar) were detected.

2.2 Induction of Enzymes through Media Modification

For this induction research, YEMEA[a] plates with different carbon sources instead of glucose and inducers were made. Glucose was a common form of additional carbon source, other carbohydrates, such as fructose and maltose, were used in here to replace glucose. Additional nitrogen sources, such as urea and amino acids, and metals, such as cobalt and ion, were also used (Table 3). Some inducers, such as cobalt and urea, were combined together. Moreover, some inducers were added to YEMEA plates in different concentrations, i.e. 0.4% and 0.7% methacrylamide.

The plates were inoculated with *R. rhodochrous DAP* 96253, incubated at 30°C for seven days, and then enzyme activities were examined by colorimetric assay.

The cells scraped from YEMEA plates with CoU were used as an inoculum for fermentation. The optimal conditions for enzyme induction of the fermentation grown cells were also detected. And both the fermented cells and cells scraped from plates were used for the stability study and fruit ripening study.

YEMEA[a] (Basic Medium):

1. Yeast Extract 4 g
2. Malt Extract 10 g
3. Glucose 4 g
4. H_2O (DI) make to 1.0 liter
   For agar, add 20 g Agar per 1.0 liter
   For agar plates, aseptically dispense 25 ml per plate

Table 3. Different inducers used for preparing YEMEA plates for induction experiment

	Inducers (Abbreviations)	Concentrations	Manufacturers
Carbon source	Glucose (G)	1, 2, or 4 g/L	Sigma-Aldrich St. Louis, MO
	Fructose (F)		
	Maltose (M)		
	Maltodextrin (MD)		J.T. Baker Phillipsburg, NJ
	Sucrose (S)		
	Trehalose (T)		
Nitrogen source	Urea (U)	125 mM	Fisher Scientific Fair Lawn, NJ
	Potassium nitrate (KNO_3)	250 mM	Mallinckrodt St. Louis, MO
	Glutamine (Gln)	1 g/L	Sigma-Aldrich St. Louis, MO
	Glutamic acid (Glna)	1 g/L	
	Asparagine (Asn)	1 g/L	
	Serine (Ser)	1 g/L	
Metal	Nicole chloride (Ni)	200 μM	Sigma-Aldrich St. Louis, MO
	Ion chloride (Fe)	200 μM	Mallinckrodt, St. Louis, MO
	Lead chloride (Pb)	100 μM 200 μM	
	Cadmium chloride (Cd)	100 μM	
	Cobalt chloride (Co)	200 μM	J.T. Baker, Phillipsburg, NJ
	Potassium cyanide(KCN)	50 μM 100 μM 150 μM	
	Zinc sulfate (Zn)	100 μM 200 μM	
Other	Methacrylamide (MeAMD)	4 g/L 7 g/L	Sigma-Aldrich, St. Louis, MO

2.3 *Rhodococcus rhodochrous* DAP 96253 Fermentation

Twenty grams packed wet weight of *Rhodococcus rhodochrous* DAP 96253 cells from YEMEA plates with CoU were scraped into 40 ml YEMEA broth as the inoculum. A Biostat C vessel (20 L) was cleaned and rinsed by dd H_2O and assembled based on the manual. 9.1 L dd H_2O was added to the vessel, which was steam sterilized. The tubings (Masterflex, Vernon Hills, IL) used in fermentation were sterilized. After sterilizing and assembling the vessel, 1.5 L R_3A (10×), 50 ml 0.23 M cobalt chloride, and 400ml 4.125 M urea were pumped into the vessel via a Masterflex pump (Niles, I L). The pH of the culture medium was set to 7.0 using HCl and

NaOH. Temperature was set at 30°C, and stir speed was set at 250 rpm. After calibration of the oxygen concentration, the *R. rhodochrous* DAP 96253 inoculum was inoculated into the vessel. According to a specific feed rate of YEMEA media (2×) for 12 hours, another 100 ml 0.23 M cobalt chloride was added into the vessel. After 48 hours fermentation, the cells were pumped directly from the 20 L Biostat C vessel into a Carr Powerfuge Pilot Separation System (Cuyahoga Falls, OH) using a Masterflex pump head with Master flex tubing (06404-24) at a speed of 400ml/min. The powerfuge was set to 11,500 rpm. When done, cells were scraped from the bowl into plastic cups, and stored in appropriate conditions for future use.

Table 4. Media *for Rhodococcus* fermentation

Table 4.a. R_3A Components

R3A Component	1× Amount (g) per Liter	10× Amount (g) per Liter	Manufacturer
Casamino Acid	0.99	9.9	Bacto, Sparks, MD
Yeast Extract	0.99	9.9	
Soluble Starch	0.99	9.9	Difco, Sparks, MD
Glucose	0.99	9.9	Sigma-Aldrich, St. Louis, MO
Sodium Pyruvate	0.5	5.0	
K_2HPO_4	0.6	6.0	Fisher Scientific, Fair Lawn, NJ
$MgSO_4$	0.1	1.0	
Cottonseed Hydrolysate	0.99	9.9	Marcor, Carlstdt, NJ

Table 4.b. YEMEA Components

YEMEA Component	1× Amount (g) per Liter	2× Amount (g) per Liter	Manufacturer
Yeast Extract	16	32	Bacto, Sparks, MD
Dextrose	26.6	53.2	J.T. Baker, Phillipsburg, NJ
Maltose	64	128	Sigma-Aldrich, St. Louis, MO
Glycerol	8	16	
Cottonseed Hydrolysate	3.9	7.8	Marcor, Carlstdt, NJ

Table 4.c. Acid, Base, and Antifoam Components

	Concentration	Manufacturer
NaOH	2 N	Fisher Scientific, Fair Lawn, NJ
HCl	1 N	Mallinckrodt, St. Louis, MO
Antifoam	20%	Sigma-Aldrich, St. Louis, MO

2.4 Determination of Enzyme Activity

Based on detecting the production of ammonia, NHase, amidase, and cyanidase activity were assayed using a modified method by Fawcett and Scott (1960) (Table 5, Fig 8). Also measuring the ammonia production, ACC deaminase assay was done based on the method developed by Nagasawa and Yagi (1966) (Table 5, Fig 8). βCAS-like enzyme activity was quantified using the method of Ezzi and Lynch (2002), based on measuring the H_2S production with the substrates potassium cyanide and cysteine (Table 5, Fig 9).

One unit of enzyme was defined as the conversion of 1 μM of accordant substrate per minute per mg dry weight of cells at room temperature, pH 7.4. Enzyme activity was determined by adding certain amount of cells to substrate. After certain reaction time, cells were removed by centrifuge, and the reaction was further terminated by acidifying the sample with 2 N H_2SO_4. Before the color reaction, detecting ammonia or hydrogen sulfide, the cell-free sample was neutralized with 2 N NaOH. After the color reaction, the absorbance was read at 630 nm via a Victor Multilabel Counter Reader (Wallac, Turku, Finland). The detailed procedures for assay and preparation of solutions were listed below (Table 5, Fig 8-9).

Table 5.NHase, amidase, cyanidase, ACC deaminase, and βCAS assays

Assay	NHase	Amidase	Cyanidase	ACC deaminase	βCAS-like enzyme
Substrate	5000 ppm Acrylonitrile (Sigma-Aldrich, St. Louis, MO)	1000 ppm Acrylamide (Sigma-Aldrich, St. Louis, MO)	50 ppm KCN (J.T. Baker, Phillipsburg, NJ)	100 ppm ACC (Sigma-Aldrich, St. Louis, MO)	0.05 M KCN* and 0.01 M L-cysteine* (Sigma-Aldrich, St. Louis, MO)
Gas Production	Ammonia	Ammonia	Ammonia	ammonia	H_2S
Reagents adding in order	1. 2 ml Sodium phenate** 2. 3 ml 0.01% Sodium nitroprusside (Mallinckrodt, St. Louis, MO) 3. 3 ml 0.15% sodium hyprochloride (Clorox, Oakland, CA)			1. 1 ml Phenol reagent*** 2. 7 ml 0.1 M Na_2HPO_4 buffer (pH 9.85) (Fisher Scientific, Fair Lawn, NJ) 3. 0.5 ml 0.005% Sodium nitroprusside 4. 0.5 ml 5% Sodium hyprochloride.	1. 0.5 ml 0.03 M $FeCl_3$**** 2. 0.5 ml 0.02 M N,N-dimethy-p-phenylenediamine sulfate***** (Fisher Scientific, Fair Lawn, NJ)

*: prepared in 0.1 M Tris-HCl (pH 8.5) (J.T. Baker, Phillipsburg, NJ).
**: 25 g phenol (Sigma-Aldrich, St. Louis, MO) was melted at 50°C, followed by adding 800 ml ddH_2O and 78 ml 4 N NaOH.
9 ml Substrate+ 1 ml cell suspension (40 mg cells suspended in 1 ml PB)
***: 0.5 g phenol and 0.2 g NaOH in 10 ml ddH_2O
****: prepared in 1.2 M HCl.
*****: prepared in 7.2 M HCl.

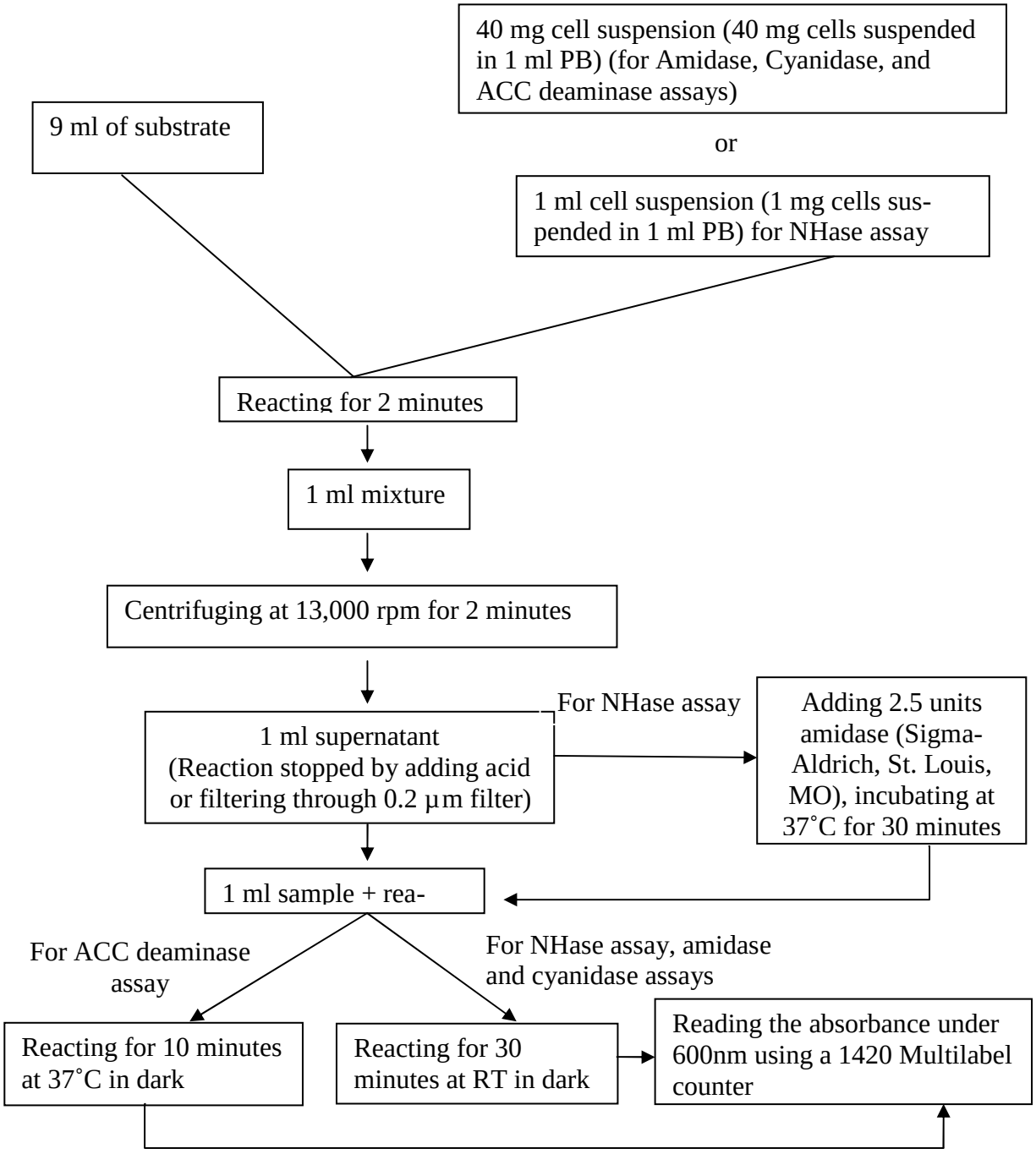


Figure 8. Procedures of NHase, amidase, cyanidase, and ACC deaminase assays

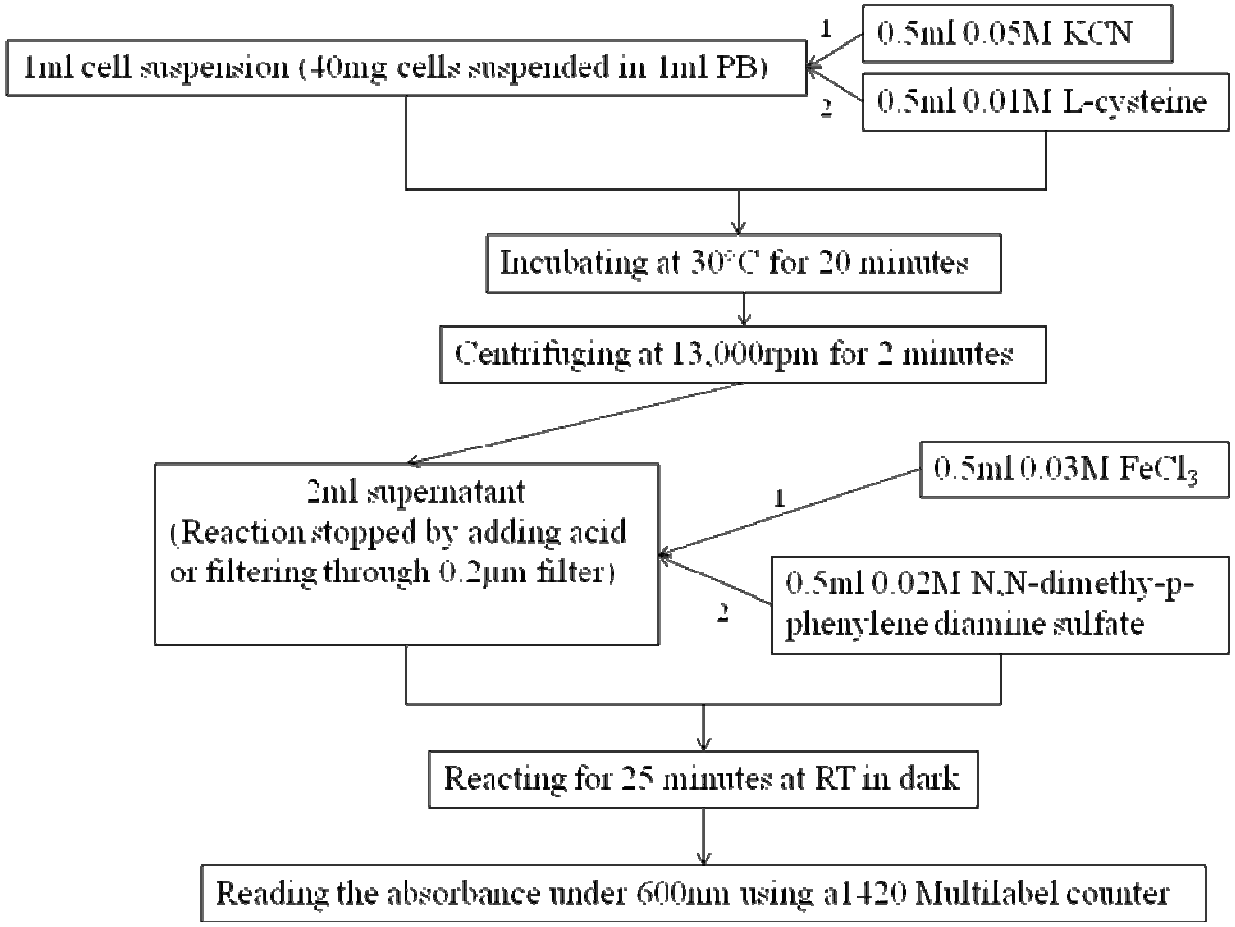


Figure 9. Procedures of βCAS assay. 1: first added in; 2: second added in

2.5 Stability Study

2.5.1 Storage Buffers Preparation

Different phosphate-sugar buffers[b] (PB) were prepared. 40 mg packed wet weight of *R. rhodochrous* DAP 96253 cells were scraped from YEMEA plates with CoU, and then suspended with 1ml of different buffers. :

phosphate-sugar buffer[b] (PB):

1. 25 mM sodium phosphate monobasic dehydrate (Fisher Scientific, Fair Lawn, NJ)
2. 25 mM sodium phosphate dibasic heptahydrate (Fisher Scientific, Fair Lawn, NJ)
3. Adjusting pH to 7.4 to make phosphate buffer (PB)
4. Adding selected sugar (0.25 M or 0.5 M maltose; 0.25 M or 0.5 M sucrose, 0.25 M or 0.5 M glucose, 18% or 9% maltodextrin) with certain concentration to PB to make phosphate sugar buffer

2.5.2 Thermostability of Enzymes with Different Storage Buffers

R. rhodochrous DAP 96253 cells with 1ml of different buffers were incubated at 4°C, 15°C, RT, 37°C, and 55°C. As another *rhodococcal* strain, *R. rhodochrous* DAP 96622 cells with 1 ml of 50 mM PB were incubated at 4°C, 15°C, RT, 37°C, and 55°C. After 30 minutes, cells with buffers were taken out and enzymes activities were assayed at room temperature.

2.5.3 Effect of Storage Buffers at Different Temperatures

R. rhodochrous DAP 96253 cells with 1 ml of different buffers were stored at -20°C, 4°C, 15°C, RT, 37°C, and 55°C. At different time periods, enzymes activities were measured to determine the protection effects on enzymes by different sugars. The NHase activity, amidase activity, ACC deaminase activity, cyanidase activity, and βCAS-like enzyme activities of *R. rhodochrous* DAP 96253 cells prior to storage were measured as the control.

2.5.4 Effect of Drying at Room Temperature on Enzyme Stability

Five grams packed wet weight of *R. rhodochrous* DAP 96253 cells were suspended with 20 ml of different storage buffers, and then poured into one side of a Petri dish. The Petri dishes with cell solutions were placed at room temperature on the bench. At certain intervals, the cells were resuspended with PB in the Petri dish, and then cells were used to do the five enzyme assays for enzyme activity and stability.

2.5.5 Osmostability of Enzymes in R. rhodochrous DAP 96253 Cells with Different Storage Buffers

R. rhodochrous DAP 96253 cells with 1ml of different buffers were dried by vacufuge (Eppendorf, Westbury, NY) at -30°C for 9 hours. The dried cells were stored at -20°C, 4°C,

15°C, RT, 37°C, and 55°C. At certain intervals, the cells were resuspended with dd H_2O, and then the five enzymes activities and stabilities were measured.

2.6 Immobilization

2.6.1 Calcium Alginate Immobilization (Beads)

Cells immobilized in calcium alginate were prepared based on a modified method of Wu and Li (2002). Twenty-five grams of 2% sodium alginate (in dd H_2O) were heated at 70ºC until the solution turned clear. Five grams packed wet weight of *R. rhodochrous* DAP 96253 cells were scraped from either GCoU plates or fermented cells. The suspension was mixed thoroughly with the sodium alginate. The mixture was then pipetted slowly through a 1 ml pipette tip (VWR, Radnor, PA) into a solution of 2% calcium chloride (Fisher Scientific, Fair Lawn, NJ). The *R. rhodochrous* DAP 96253 immobilized beads were cured in the calcium chloride solution immediately, sitting for 60 minutes, and then washed with 8% NaCl and stored at 4ºC.

Lyophilized cells and lyophilized calcium alginate beads were prepared by Freeze drying system/ Lyph Lock 4.5 (Labconco, Kansas City, MO) at -40 ºC for 24 hours.

2.6.2 Crossing-linking R. rhodochrous DAP 96253 Cells with Glutaraldehyde and Polyethylenimine (GA-PEI)

Three grams of *R. rhodochrous DAP* 96253 cells were scraped from YEMEA plates and suspended with 25ml ddH_2O, approximately 500 mg of polyethylenimine (PEI) (Sigma-Aldrich, St. Louis, MO) solution was added to the cell suspension, with stirring until homogeneous. The cell-PEI suspension was then placed on ice for 30 minutes to cool to 4ºC. After cooling, 1 ml of 25% glutaraldehyde (Sigma-Aldrich, St. Louis, MO) solution was added to the cell-PEI suspension. After flocculation was achieved, flocculated materials were collected by centrifuging at

10,000 rpm for 5 min, or by filtering through the cheesecloth wipe (VWR, Radnor, PA). The glutaraldehyde and polyethylenimine (GA-PEI) immobilized cells were removed onto a glass plate or aluminum foil ; and spread out to form a layer with uniform thickness, once dried, the GA-PEI cells were collected in a falcon tube (Falcon, Franklin Lakes, NJ), stored at room temperature.

2.6.3 Wax Immobilization

Different wax emulsions were prepared based on a modified method by Hagenmaier (2004). Carnauba wax emulsions (CWE) were prepared as follows. 40 g carnauba wax (Sigma-Aldrich, St. Louis, MO), 15g oleic acid (Sigma-Aldrich, St. Louis, MO), 15 g water, and 0.15 g 5% antifoam were heated in boiled ddH_2O for 10 minutes. 28 g 8% NH_4OH (J.T. Baker, Phillipsburg, NJ) was very slowly added into the wax mixture. After that, 160 ml hot ddH_2O (about 85°C) was slowly added to make the wax emulsion. The final mixture was stirred for 2 minutes. The wax emulsion was cooled to room temperature for future use. The improved carnauba wax emulsion was prepared similar to the CWE; the difference was using NaOH as emulsifier instead of NH_4OH. Mineral oil emulsion (MOE) was prepared the same as CWE. Since mineral oil (Sigma-Aldrich, St. Louis, MO) was liquid, MOE was prepared at room temperature.

The 5 g packed wet weight of *R. rhodochrous* DAP 96253 cells were scraped from YEMEA plates with cobalt and urea and transferred to 10 g wax emulsions, under continuous stirring at a low speed to mix thoroughly. The mixture was poured into a glass plate. Wax immobilized *R. rhodochrous* DAP 96253 whole cells were prepared when the mixture dried. The five enzyme activities were measured to test the effects on the stability of the different immobilized cells. The supernatants in the enzyme assays were filtered instead of centrifuged. The wax emulsions were also tested as controls.

2.6.4 Storage Stability of Immobilized Cells

For storage stability studies, the Ca-alginate (wet and lyophilized), GA-PEI, and wax immobilized cells were kept at 4°C and room temperature. The enzyme activities of different immobilized cells were measured at certain intervals. Fresh preparations of immobilized cells and fresh cells were taken as controls for each assay.

2.7 Effects of *Rhodococcus* on Fruit Ripening

Bananas and peaches were selected for this fruit ripening study. Bananas were purchased commercially (Chiquita#4011, Guatemala). Peaches were provided through the auspices of the Georgia Peach Council, with special thanks to Robert Dickey, III at Dickey Farms (Musella, GA) and Bill McGehee at Pearson Farms (Fort Valley, GA).

2.7.1 Comparison of Three Rhodococcal Strains

Three *rhodococcal* strains: *R. rhodochrous* DAP 96253 and *R. rhodochrous* DAP 96622 cells from GCoU plates, and *R. erythropolis* ATCC 47072, were used for delaying fruit ripening. Five grams packed wet weight of each strain were suspended with 20 ml M9 salts working solution[1]. The cell suspension was poured into a petri dish, non-contact with 1kg bananas, in a sealed plastic 3.85 L container. Periodically, pictures were taken to compare the effects of different strains on fruit ripening.

M9 salts working solution[1]:

1. 64 g Na_2HPO_4, 15 g KH_2PO4, 2.5 g NaCl, 2 g Glucose, and 5 g NH_4Cl
   Adjusting to 1000 ml M9 salts concentration with dd H_2O
2. 200 ml M9 salts concentration, 2 ml of 1 M $MgSO_4$, 100 µl of 1 M $CaCl_2$
   Adjusting to 1000 ml M9 salts working solution with dd H_2O

2.7.2 R. rhodochrous DAP 96253 Cells Grown with Different Inducers

Four different YEMEA plates: uninduced (G), GCo, GCoU, and GU, and fermentation grown cells were selected for fruit ripening experiment. 5 g packed wet weight cells scraped from these plates were suspended with 10 ml M9 salts solution. The effects of other buffers, such as PB and PB with trehalose, were also tested. Cell suspension was poured into an empty plate with bananas in a sealed 4.4 L box. Bananas sealed in a box were used as control. Pictures were taken at defined intervals to compare the effects of *R. rhodochrous* DAP 96253 on fruit ripening.

2.7.3 Immobilized R. rhodochrous DAP 96253 Cells

The Ca-alginate beads, GA-PEI cells and wax immobilized cells, which equal to 5 g packed wet weight *R. rhodochrous* DAP 96253 cells were placed with fruits (without contacting the fruits) in sealed containers.

To examine the effect of wax coating with cells on fruit ripening (contact with the fruits), 10 g of different wax emulsions were diluted to different wax concentration, from 5% to 20%. Five grams *R. rhodochrous* DAP 96253 were scraped from GCoU and transferred to the diluted wax solution. This thoroughly mixed suspension was used to spray the fruits and for dipping fruits. The fruits with wax-cell coatings were placed in sealed boxes. Wax emulsions without cells were also used to spray and for dipping fruits as controls. Fruits in a sealed box were used as the control. Pictures were taken of the fruits after defined intervals.

2.7.4 Fermented Cells on Fruit Ripening

Fermented *R. rhodochrous* DAP 96253 cells were harvested and used for the fruit ripening experiments and immobilization. Moreover, the effect of fermented cell paste without any

buffer on fruit ripening was determined as well. The set up protocols were similar as described as above.

2.7.5 Effects of Different Amount of Catalysts on Fruit Ripening

Different amounts of live catalysts and immobilized cells were placed with fruits in sealed boxes to test the effects on fruit ripening. Immobilization materials without catalyst were placed with fruits in sealed boxes as controls.

2.7.6 Standard for Various Stages of Bananas Ripening

Other than the appearance of bananas, starch transformation and peel thickness were used as standard to identify the various stages of bananas ripening. One slice of banana was cut in the middle of the banana in each ripening stages, four drops of stabilized Gram iodine (Difco, Sparks, MD) was added on the slice, separately. After 15 minutes, the excess dye was gently rinsed out by ddH_2O. The thicknesses of peels cut in the middle of bananas in various stages were also measured.

2.8 Electron Microscopy

Immobilized cells were imaged using a Leo 1450 VP SEM. The glutaraldehyde fixed cells were provided to Dr. Robert B. Simmons.

2.9 Gas Chromatography (GC)

2.9.1 GC Method 1

Rhodococcal cells scrapped from plates [1 g packed wet weight, *R. rhodochrous* DAP 96253 or *R. rhodochrous* DAP 96622 scraped from Co U, *R. erythropolis* 47072 scraped from NA, and *R. rhodochrous* DAP 96253 scraped from NA (7 days)], and glutaraldehyde immobilized *R. rhodochrous* DAP 96253 (CoU) cells were added to the edge of amber vials (20 ml)

(Thermo Scientific, Miami, OK), containing 2 ml 100 ppm KCN, 15 ml ethylene (100 ppm in air) added through a Miniert valve (VICI Precision Sampling, Baton Rouge, LA). The following groups were prepared in 20 ml amber vials: 2 ml 100 ppm KCN and 15 ml ethylene in air only; 1 g cells only; 1 g cells with 15ml ethylene; 1 g cells with 2ml 100 ppm KCN; 1 g cells with 4.5 g ripened bananas; 4.5 g ripened bananas only; glutaraldehyde immobilized cells equaled to 1 g cells from plates were used. Vials were incubated for 20hrs at 30°C with agitation (130 rpm).

Head space gas analysis was conducted on samples without fruit, 500 µl of headspace gas was injected into a DB-624 column (J&W Scientific, Rancho Cordova, CA). A Perkin Elmer GC (Norwalk, CT) was used with a FID detector set to 200°C, the injector was set to 250°C, and the oven temperature program started at 80°C held for 1 min. The temperature was increased at a rate of 20 °C/min until 120°C, then held for 5 min. Carrier gas (He) was set to 10 ml/min. Attenuation was set to -5.

Head space gas analysis was conducted on samples with fruit, 500 µl of headspace gas was injected into a DB-624 column. A Perkin Elmer GC was used with a FID detector set to 200°C, the injector was set to 250 °C, and the oven temperature program started at 80°C held for 2 min. The temperature was increased at a rate of 20 °C/min until 200°C, and then held for 8 min. Carrier gas (He) was set to 10ml/min. Attenuation was set to -5.

2.9.2 GC Method 2

Rhodococcal cells scraped from plates or fermentation, lyophilized and glutaraldehyde immobilized *R. rhodochrous* DAP 96253 cells (from CoU plate or fermented) were added to the edge of amber vials (20 ml) (Thermo Scientific, Miami, OK). Bananas, in various ripening stages (1-6 scale that build up based on starch transformation), were added to the vials as controls. The following groups were prepared in 20 ml amber vials: 1 g cells only; 1 g cells with 4.5 g rip-

ened bananas; 4.5 g ripened bananas only; lyophilized or glutaraldehyde immobilized cells equal to 1 g cells were used. Vials were incubated for 20 hrs and 48hrs at 30°C with agitation (130 rpm).

Head space gas analysis was conducted on samples without fruit, 500 µl of headspace gas was injected into a DB-624 column (J&W Scientific, Rancho Cordova, CA). A Perkin Elmer GC (Norwalk, CT) was used with a FID detector set to 200°C, the injector was set to 250°C, and the oven temperature program started at 80°C held for 1 min. The temperature was increased at a rate of 20 °C/min until 180°C, then held for 6 min. Carrier gas (He) was set to 10 ml/min. Attenuation was set to -5.

2.9.3 GC Method 3

Rhodococcal cells scraped from plates or fermentation, and glutaraldehyde immobilized *R. rhodochrous* DAP 96253 cells (from CoU plate or fermented) were added to the edge of amber vials (20 ml) (Thermo Scientific, Miami, OK). Bananas, in stage 1 (1-6 scale that build up based on starch transformation), were added to the vials as controls. The following groups were prepared in 20 ml amber vials: 1 g cells only; 1 g cells with 4.5 g ripened bananas; 4.5 g ripened bananas only; 1 g glutaraldehyde immobilized cells. Vials were incubated for 20 hrs at 30°C with agitation (130 rpm). After 20 hrs, these vials were placed at room temperature without agitation, followed by insertion of SPME fibers (Carboxen/PDMS) for 1 hr.

The volatiles extracted by the fibers were analyzed by GC as followed. SPME fiber was injected into a DB-624 column (J&W Scientific, Rancho Cordova, CA). A Perkin Elmer GC (Norwalk, CT) was used with a FID detector set to 200°C, the injector was set to 250°C, and the oven temperature program started at 80°C held for 2 min. The temperature was increased at a

rate of 20 °C/min until 180°C, then held for 14 min. Carrier gas (He) was set to 10 ml/min. Attenuation was set to -5.

2.10 Fungal Inhibition Set Up

Aspergillus niger and *Penicillium spp.* (10 µl of 1×10^4 ml^{-1} spore concentration) were inoculated on 10% SAB or uninduced YEMEA (G^-) plates. Two grams of catalyst were transferred to an empty petri dish (100×15 mm). Medium size petri dishes (35×10 mm) were inoculated with spores and placed into large petri dishes (100×15 mm) containing GA-PEI catalysts (2 g). Controls were set up without catalyst, experiments were set up in duplicates. The petri dishes (100×15 mm) were parafilmed and incubated at 30°C for 48 hours and 72 hours.

3 RESULTS

3.1 Enzyme Production

3.1.1 Comparison Enzyme Production among Rhodococcal Strains

R. erythropolis ATCC 47072 can not grow as well as either *R. rhodochrous* DAP 96622 or *R. rhodochrous* DAP 96253 on YEMEA plates with CoU. Therefore cells for the enzyme assay of *R. erythropolis* ATCC 47072 were scraped from NA plates without any inducers. There was no significant difference among the three strains with regards to cyanidase, but there was a statistically significant difference in NHase. Amidase and ACC deaminase levels of *R. erythropolis* ATCC 47072 were 35% less than *R. rhodochrous* DAP 96253, which were significant. There was no NHase activity detected in *R. erythropolis* ATCC 47072 (NA) cells, however the βCAS-like enzyme level of *R. erythropolis* ATCC 47072 was twice as high as *R. rhodochrous* DAP 96253, and 57% higher than *R. rhodochrous* DAP 96622. NHase activity in *R. rhodochrous* DAP 96622 was 15.7% higher than in *R. rhodochrous* DAP 96253, which indicated a high significance, and the amidase activity was 25% lower than *R. erythropolis* ATCC 47072. Though amidase activity of *R. erythropolis* ATCC 47072 was higher than *R. rhodochrous* DAP 96622, there was no significant difference by statistical analysis. *R. rhodochrous* DAP 96253 has the highest enzymes activity among the three strains except for the βCAS-like enzyme (Table 6, Table 7).

Table 6. Enzyme activity of three *Rhodococcal* strains when grown on YEMEA plates supplemented with cobalt and urea

	NHase (units/mg cdw)	Amidase (units/mg cdw)	Cyanidase (units/mg cdw)	ACC deaminase (units/mg cdw)	βCAS-like (units/mg cdw)
R. erythropolis ATCC 47072 (NA) [a]	0	12	7	7	22
R. rhodochrous DAP 96622 (CoU) [b]	40	9	7	7	14
R. rhodochrous DAP 96253 (CoU) [c]	210	28	8	15	8

Table 7. Statistical significance of enzyme activity of three *Rhodococcal* strains

	NHase			Amidase			Cyanidase			ACC deaminase			βCAS-like		
	a	b	C	a	b	c	a	b	C	a	b	C	a	b	C
a	-	**	**	-	-	*	-	-	-	-	-	*	-	*	**
b	**	-	**	-	-	*	-	-	-	-	-	*	*	-	-
c	**	**	-	*	*	-	-	-	-	*	*	-	**	-	-

* statistically significance ($p<0.05$); ** statistically highly significance ($p<0.001$)

3.1.2 Effects of Different Compounds on Enzymes Production of R. rhodochrous DAP 96253

R. rhodochrous DAP 96253 grew on the following heavy metals: 200 µm Zn, Pb, Ni, Fe, and Co, and it tolerated 100 µm Cd, but did not grow on 200 µm Cd and 1 g/L glutamic acid. After seven days incubation at 30°C, *R. rhodochrous* DAP 96253 also tolerated 150 µm KCN, the highest concentration of KCN tested (Table 8).

R. rhodochrous DAP 96253 grown with or without inducers, produced βCAS-like enzyme. The level of βCAS-like enzyme was not affected as much as the other enzymes by additives in its growth media. When urea was added in YEMEA agar, NHase, amidase, cyanidase

and ACC deaminase significantly increased. Asparagine, methacrylamide, glutamine, Ni, Fe, and Co when used separately can slightly induce NHase, but when Co and urea were combined, the NHase activity significantly increased (Table 8), and was the highest among the inducers tested. Combining Co and U with other inducers such as $FeSO_4$, methacrylamide, and KCN, showed no significant difference in the five enzymes compared to CoU (data not shown). Methacrylamide, glutamine (Gln), asparagine (Asn), or KNO_3, separately, as supplements, increased the activities of all three enzymes: amidase, ACC deaminase, and cyanidase. The effect of asparagine on amidase was not as substantial as the other two enzymes. $FeSO_4$ increased the activities of amidase and ACC deaminase, but did not induce cyanidase. Combining urea with either KNO_3, glutamine, or Co, induced the amidase and ACC deaminase to the highest level.

Table 8. Effects of different supplementations in growth media (YEMEA) plates on enzyme production in *R. rhodochrous* DAP 96253

Inducer(s)	NHase (units/mg cdw)	AMD (units/mg cdw)	ACCD (units/mg cdw)	Cyanidase (units/mg cdw)	βCAS-like (units/mg cdw)
KCN 50 µm	0	0	0	0	9
KCN 100 µm	0	0	0	0	9
KCN 150 µm	0	0	0	0	9
0.4% Methacrylamide	7	7	15	8	10
0.7% Methacrylamide	16	17	17	9	10
Glutamine	1	12		11	8
Asparagine	2	2	14	5	10
Glutamine + Asparagine	2	13	13	12	9
Glutamine + U	20	35	9	7	8
KNO_3 250 mM	1	15	15	11	9
KNO_3 250 mM + U	1	24	20	5	9
Serine	1	1	1	1	9
$ZnCl_2$ 100 µm	0	0	0	0	8
$ZnCl_2$ 200 µm	0	0	1	0	9
$PbCl_2$ 100 µm	1	0	0	0	9
$PbCl_2$ 200 µm	1	0	0	1	8
$CdCl_2$ 100 µm	0	0	0	1	9
$NiCl_2$ 200 µM	15	0	1	0	9
$FeCl_3$ 200 µM	10	11	5	0	9
Co	12	2	0	0	9
U	32	29	23	7	9
FeU	1	30	18	9	9
CoU	220	28	18	7	8
CoU 0.4% Methacrylamide	205	28	19	8	9
CoU 0.7% Methacrylamide	205	26	19	8	8
100 µm KCN + CoU	220	18	18	6	9
200 µM Fe + CoU	230	30	13	9	9
Uninduced	1	0	0	0	9

Co: 200 µM cobalt chloride; U: 125 mM urea
Unless otherwise specified, amino acids were filter sterilized into YEMEA at a concentration of 1000 ppm

3.1.3 Effects of Sugars of the Growth Media on Enzyme Activity

Growth media containing different types of sugars had unique effects on enzyme production in *R. rhodochrous* DAP 96253 (Table 9). Adding trehalose instead of glucose in YEMEA with cobalt and urea increased all five enzyme activities: significantly increasing NHase by 28%

and amidase by 62%, but cyanidase at 29% and βCAS-like enzyme that were not significant. Maltodextrin and sucrose also can slightly increase NHase activity, but this was not significant (Fig 10-A). There was no significant difference among the sugars with regards to cyanidase activity (Fig 10-C). ACC deaminase and βCAS-like enzyme were significantly affected by maltodextrin, with a 33% and a 43% increase, respectively (Fig 10-D, E). The amidase activity was increased in the presence of the following sugars that were in the media: trehalose, sucrose, and maltose. Moreover, maltodextrin significant increased the amidase activity by 28% (Fig 10-B). Sucrose can also increase βCAS-like activity by 71%, but the difference was not significant (Fig 10-E). *Rhodococcus* grown with fructose, which has rapid metabolic rate, has lower NHase and ACC deaminase activity compared to glucose (Fig 10-A, D).

Table 9. Average enzyme activity and significance comparing to glucose by various sugars for *R. rhodochrous* DAP 96253 grown on YEMEA or on YEMEA with other sugars in place of glucose

	NHase (units/mg cdw)	Amidase (units/mg cdw)	ACC deaminase (units/mg cdw)	Cyanidase (units/mg cdw)	βCAS-like (units/mg cdw)
T	278*	47**	14	9	9
S	238	47**	10	7	12
M	217	44**	15	7	9
F	191	40**	11	7	8
MD	233	37*	16*	7	10*
G	217	29	12	7	7

* statistically significance ($p<0.05$); ** statistically highly significance ($p<0.001$)
T: trehalose; S: sucrose; M: maltose; F: fructose; MD: maltodextrin; G: glucose

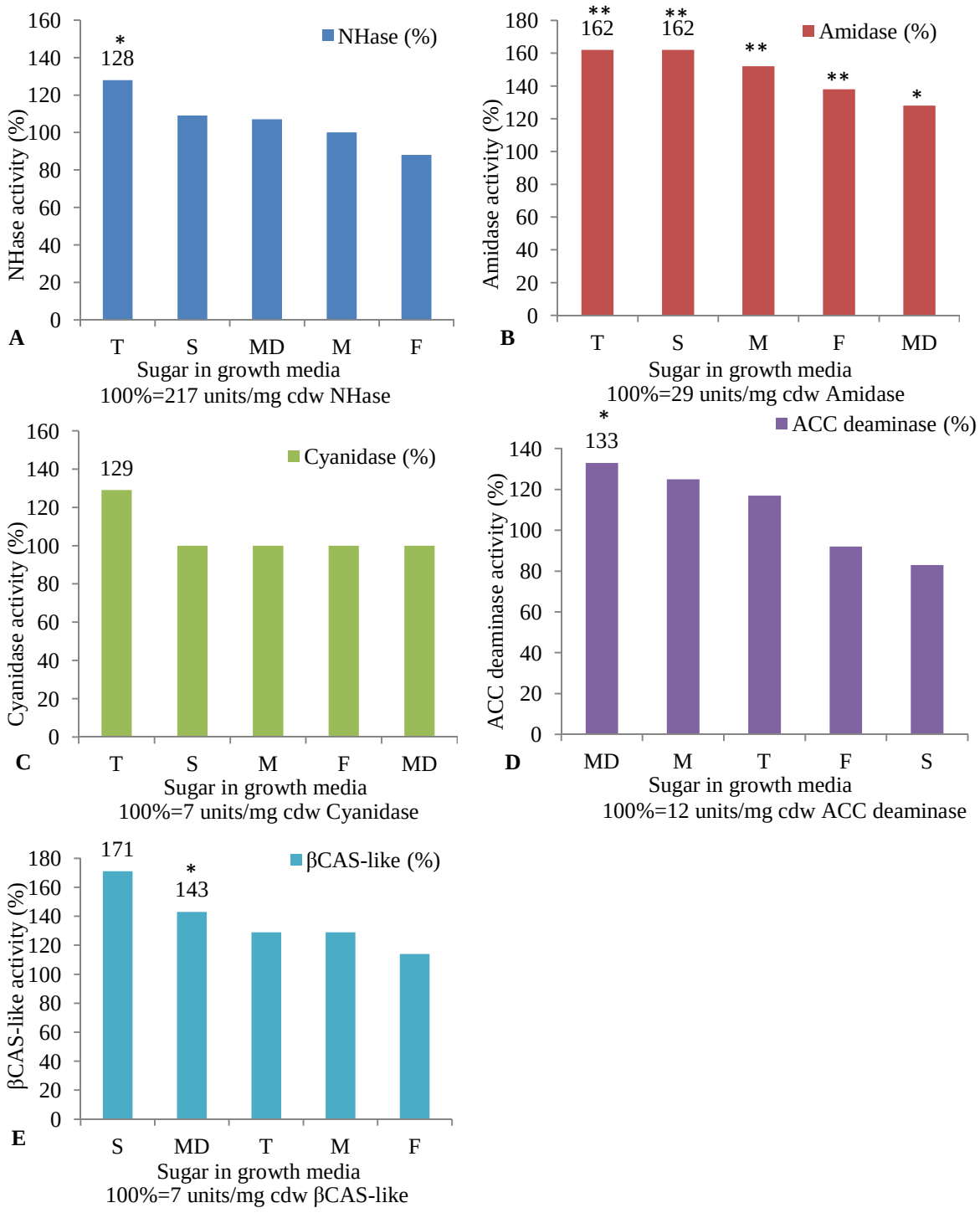


Figure 10. NHase (A), amidase (B), cyanidase (C), ACC deaminase (D), and βCAS-like (E) activity of *R. rhodochrous* DAP 96253 grown on YEMEA (CoU) plates with 4 g/L different sugars instead of 4 g/L glucose

* statistically significance ($p<0.05$); ** highly statistical significance ($p<0.001$)

F: fructose; S: sucrose; M: maltose; MD: maltodextrin; T: trehalose; enzymes activities of *R. rhodochrous* DAP 96253 grown on glucose (CoU) were used as indicators of 100%, separately

3.1.4 Effects of Different Amount of Sugars of the Growth Media on Enzyme Activity

Cells grown on different sugar-based YEMEA had different enzymes levels, and trehalose could induce most of the enzymes. Therefore, the effects of different amounts of glucose and trehalose on enzyme production in *R. rhodochrous* DAP 96253 were analyzed (Fig 11). *R. rhodochrous* DAP 96253 could grow well with a low amount of sugar, and a lower amount of sugar (2 g/L or 1 g/L instead of 4 g/L) in YEMEA media did not affect its cyanidase, ACC deaminase and βCAS-like activity. The activities of cyanidase, ACC deaminase and βCAS-like enzyme were slightly higher when cells were grown with a low amount of sugar than with a higher amount of sugar, but the effect was not significant (Fig 11-C, D, E). When the amount of sugar in the media increased, the NHase activity of *R. rhodochrous* DAP 96253 increased. The NHase activity of cells grown on 1 g/L sugar, either glucose or trehalose, were 40% which was the same as cells grown on 4 g/L of sugars, however with 2 g/L sugar, the NHase activity increased to approximately 65% (Fig 11-A). The influence of sugar on amidase was not as excessive as NHase, and cells grown with 1 g/L sugar could have approximately 85% amidase activity as with 4 g/L sugar. It was shown that amidase activity slightly increases when the amount of trehalose increases (Figure 11-B).

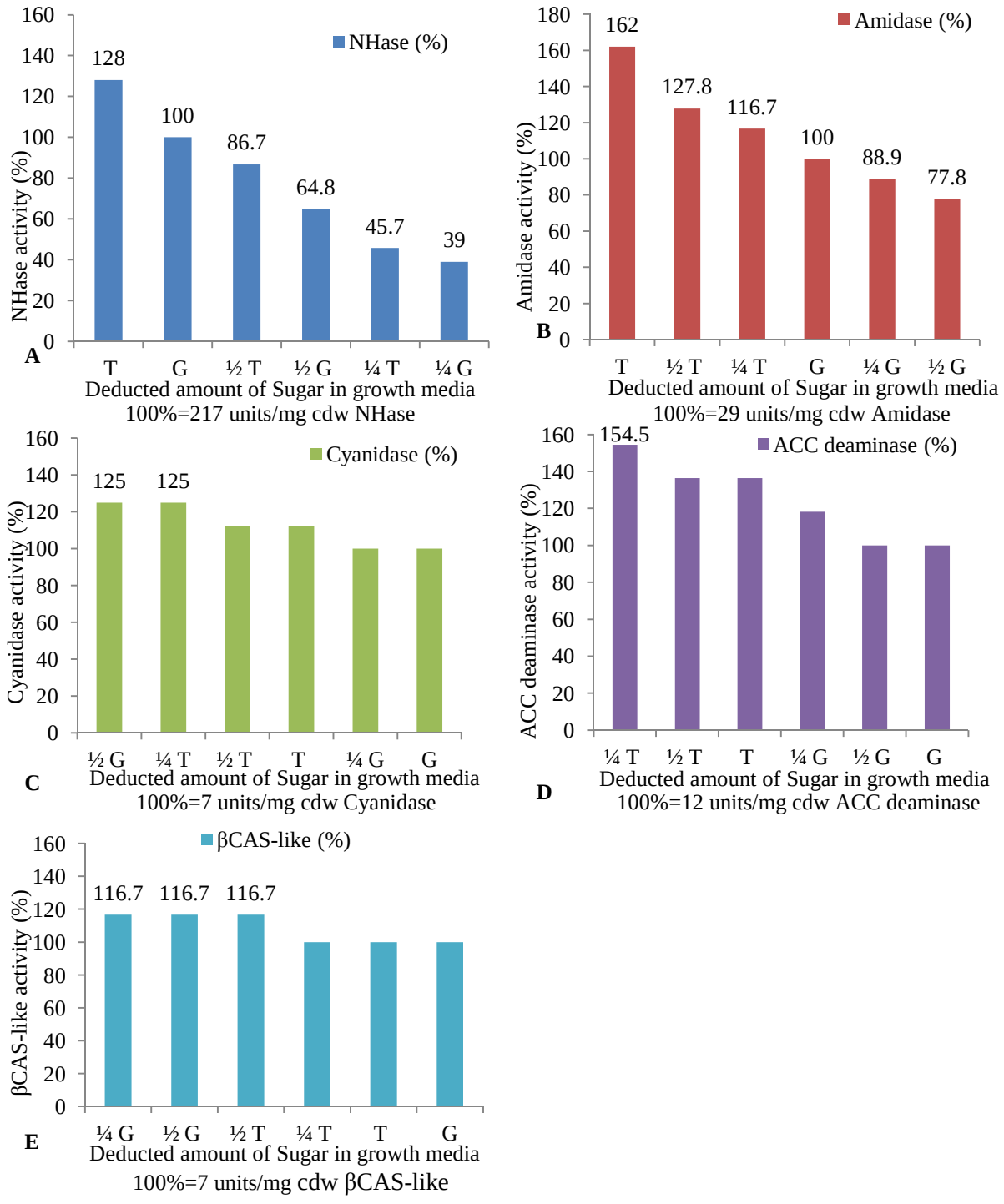


Figure 11. Enzyme activities of *R. rhodochrous* DAP 96253 grown on YEMEA (CoU) plates with deducted amount of sugars instead of 4 g/L glucose

T: 4 g/L trehalose; G: 4 g/L glucose; ½ T: 2 g/L trehalose; ¼ T: 1 g/L trehalose; ½ G: 2 g/L glucose; ¼ G: 1 g/L glucose; enzymes activities of *R. rhodochrous* DAP 96253 grown on glucose (CoU) were used as indicators of 100%, separately

3.1.5 Effects of Different Mixture of Sugars of the Growth Media on Enzyme Activity

A mixture of sugars in YEMEA plates have different effects on enzyme production of *R. rhodochrous* DAP 96253 (Fig 12). All the sugar mixtures tested showed a higher enzyme activity of the five enzymes than glucose, however, the higher enzyme activity was not simply equal to the additive of the induction by a single sugar. With a 2 g/L trehalose and 2 g/L sucrose mixture, cells showed highest NHase activity, 35% more than 4 g/L glucose, and more than sucrose and trehalose when added separately (Fig 10-A, Fig 12-A). With 2 g/L trehalose and 2 g/L glucose, cells showed the highest amidase activity, 48% more than 4 g/L glucose (Fig 12-B). Cells grown with 2 g/L trehalose and 2 g/L sucrose did not show as high of an amidase as 4 g/L trehalose or sucrose (Fig 10-B, Fig 12-B). Cells grown with 4 g/L glucose and 2 g/L trehalose showed the highest cyanidase activity, 25% more than 4 g/L glucose, while with 2 g/L trehalose and 2 g/L maltose mixture showed 50% higher ACC deaminase and βCAS-like activities than normal glucose (Fig 12-C, D, E).

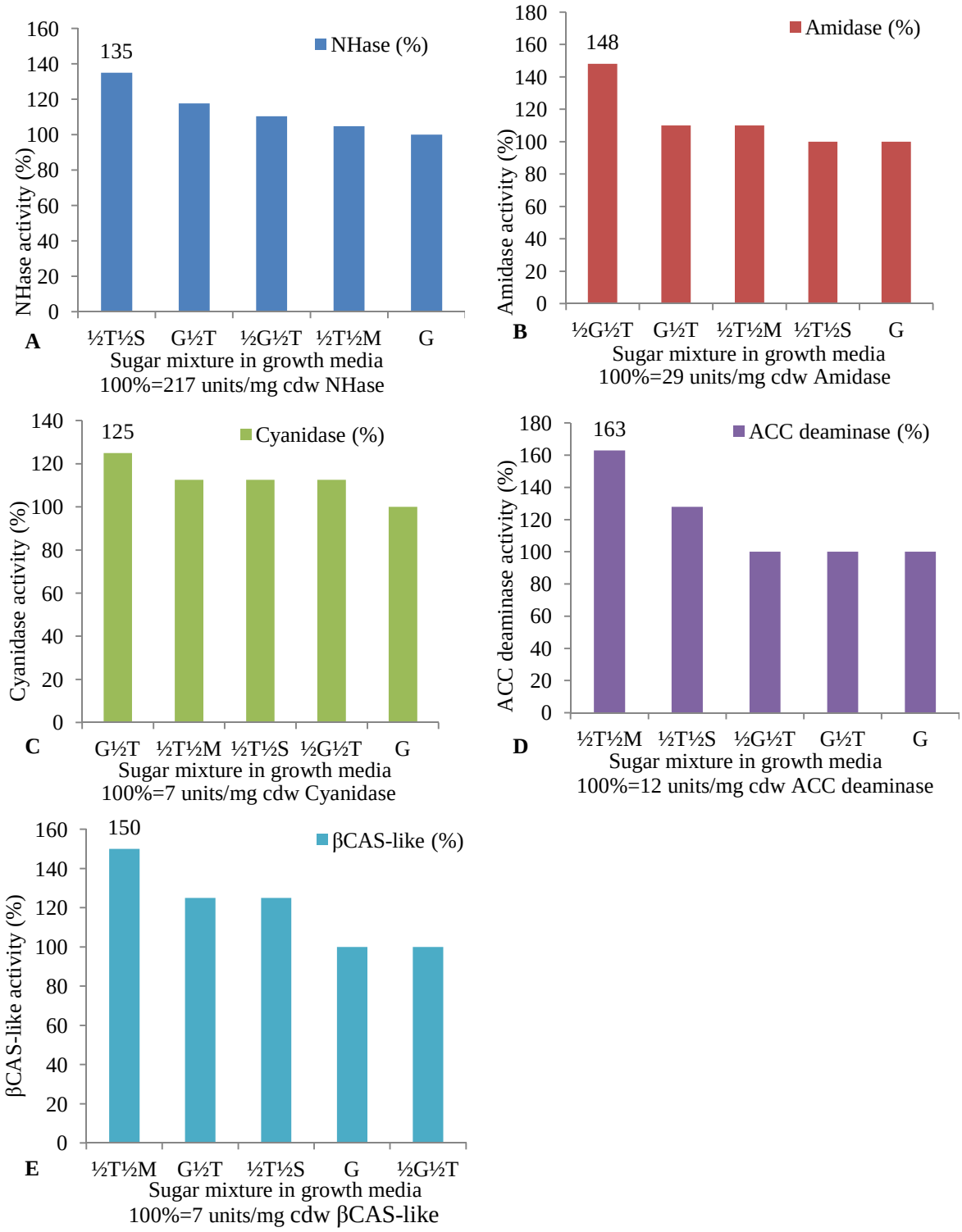


Figure 12. Enzyme activities of *R.rhodochrous* DAP 96253 grown on YEMEA (CoU) plates with different sugar mixtures instead of 4 g/L glucose

G: glucose; ½ T ½ M: 2 g/L trehalose and 2 g/L maltose; ½ T ½ S: 2 g/L trehalose and 2 g/L sucrose; ½ G ½ T: 2 g/L glucose and 2 g/L trehalose; G ½ T: 4 g/L glucose and 2 g/L trehalose; enzymes activities of *R. rhodochrous* DAP 96253 grown on glucose (CoU) were used as indicators of 100%, separately

3.2 Thermostability and Osmostability of Enzymes (Whole Cell)

3.2.1 Thermostability of Enzymes in R. rhodochrous DAP 96253 Whole Cells with Different Sugar Buffers

3.2.1.1 NHase

In *R. rhodochrous* DAP 96253 whole cells scraped from YEMEA CoU plates suspended in different sugar buffers, and incubated at various temperatures for 30 minutes, showed different NHase thermostability with different sugar buffers (Fig 13). At 4°C, all the sugars except maltose protected NHase activity better than PB. Maltodextrin and glucose had the best effects on NHase stability (Fig 13-A). At 15°C, cells with sucrose and maltodextrin had the best effects on NHase stability while cells exposed to other sugars had similar effects as PB (Fig 13-B). At 37°C, cells with trehalose and sucrose showed the highest NHase activity whereas cells with maltose and glucose contained lower NHase activity than PB. Cells with maltodextrin have similar NHase activity as PB (Fig 13-C). At 55°C, maltodextrin had the best effect on maintaining NHase activity, and 0.5 M trehalose and 0.25 M sucrose also protected NHase in *Rhodococcus* whole cells (Fig 13-D). For most sugars except maltose, NHase at 15°C was less stable than 4°C and 37°C (Fig 13-A, B, C). NHase activity of cells with sugar buffers decreased as the temperature reached 55°C (Fig 13-D). The concentration of sugar in the buffer did not play a critical role in NHase protection. Trehalose, maltodextrin, and sucrose showed better protection ability for NHase activity when incubated for 30 minutes at all four temperatures, whereas maltose and glucose were less effective than PB.

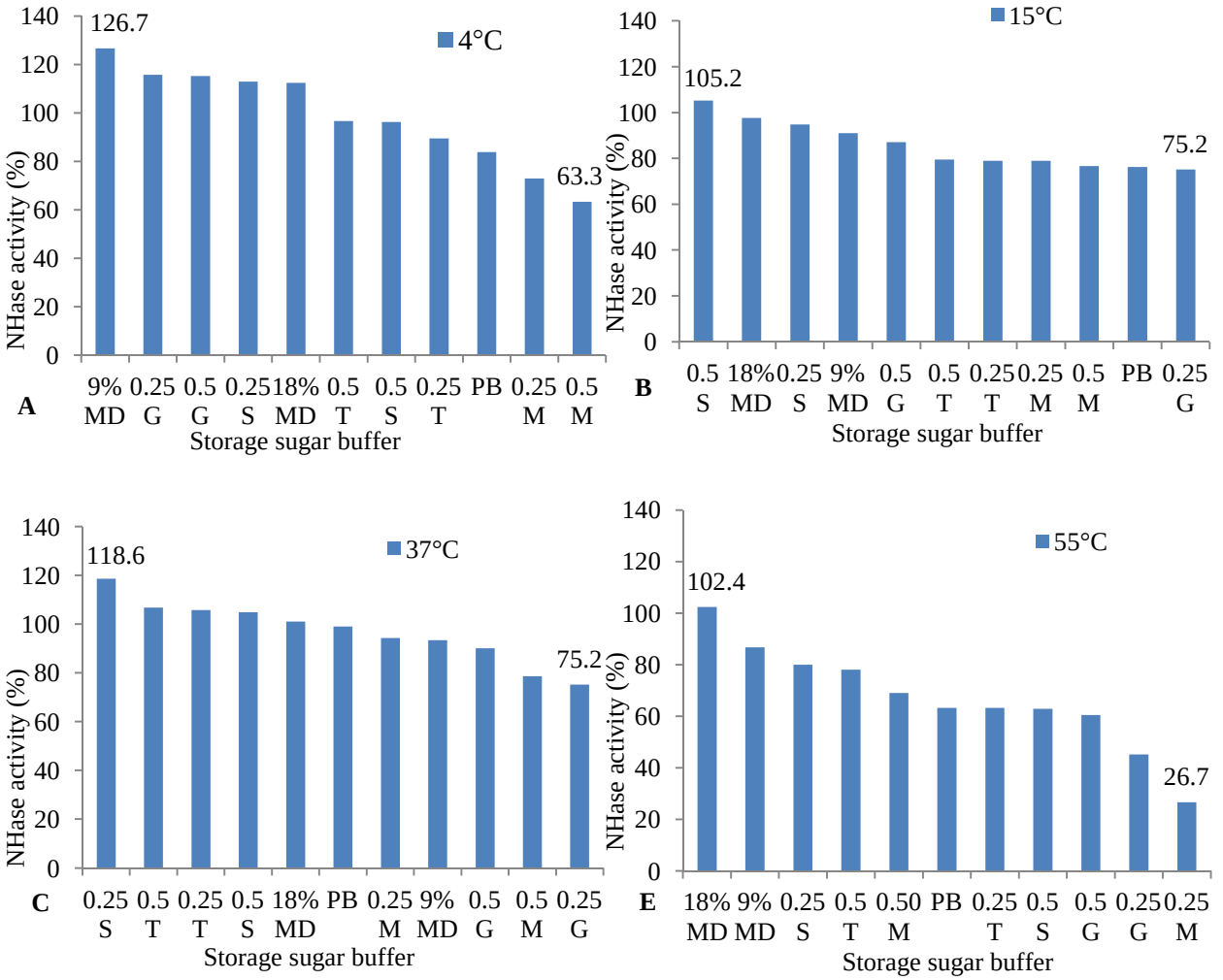


Figure 13. NHase thermostability in *R. rhodochrous* DAP 96253 whole cells with different sugar buffers incubated at various temperatures for 30 minutes. 100% = 220 units/mg cdw NHase at pH 7.4, RT.
G: glucose, F: fructose, S: sucrose, M: maltose, MD: maltodextrin, T: trehalose; PB: 50 mM phosphate buffer; 0.25/0.5: 0.25 M/0.5 M concentration of sugars (molar); 9%/18%: concentration of MD (w/v percentage)

3.2.1.2 Amidase

The protection of sugars for amidase was not as significant as NHase. With PB, amidase was stable at 4°C, 15°C, and 37°C (Fig 14-A, B, C), but when the temperature increased to 55°C, cells from the highest sugar concentrations showed higher amidase activity than PB (Fig 14-D). At 4°C, PB and 18% maltodextrin showed the highest protection ability on amidase, however,

the amidase activity of cells with maltodextrin decreased rapidly when the temperature increased (Fig 14). Cells with 0.5 M glucose showed the highest amidase activity at 15°C, 37°C, and 55°C, and relatively high amidase at 4°C (Fig 14). *R. rhodochrous* 96253 cells that were stored with neither maltose nor sucrose showed high amidase activity after incubation at variable temperatures for 30 minutes (Fig 14).

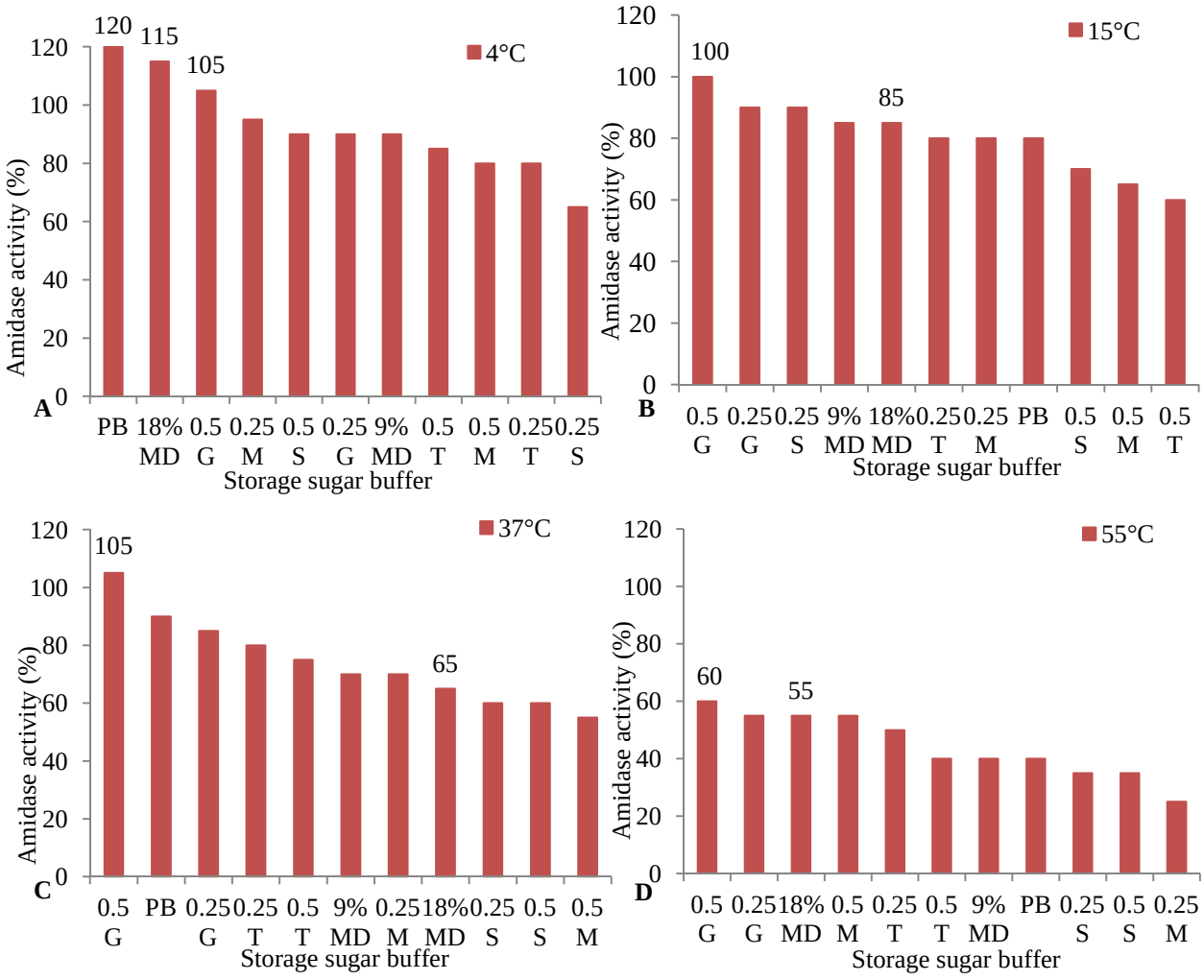


Figure 14. Amidase thermostability in *R. rhodochrous* DAP 96253 whole cells with different sugar buffers incubated at various temperatures for 30 minutes. 100% = 29 units/mg cdw Amidase.
G: glucose, F: fructose, S: sucrose, M: maltose, MD: maltodextrin, T: trehalose; PB: 50 mM phosphate buffer; 0.25/0.5: concentration of sugars (molar); 9%/18%: concentration of MD (w/v percentage)

3.2.1.3 Cyanidase

Sugars in storage buffer did not show high protection ability for cyanidase, which was stable for cells with 50 mM PB. In whole cells stored with PB incubated at 4°C, 15°C, 37°C, and 55°C for half an hour, there was no loss of cyanidase activity (Fig 15). With sugar buffers, still there was no significant loss of activity at 4°C, 15°C, and 37°C, and a slight decrease of cyanidase activity at 55°C, since the units of cyanidase activity was around 7 units/mg cdw before storage (Fig 15). Maltodextrin did not show the same protection for cyanidase as NHase at lower temperatures 4°C and 15°C (Fig 13-A, B; Fig 15-A, B).

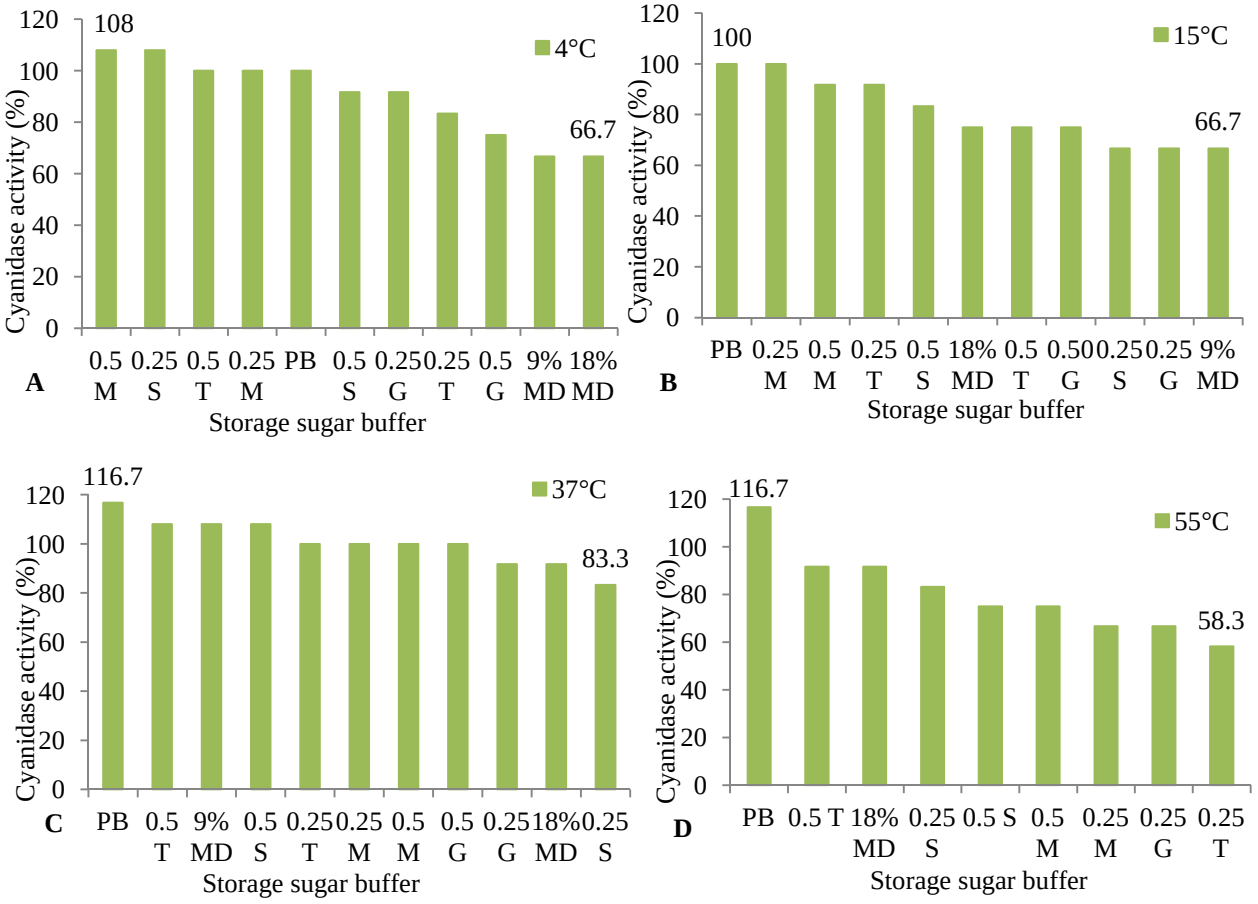


Figure 15. Cyanidase thermostability in *R. rhodococcus* DAP 96253 whole cells with different sugar buffer incubated at various temperatures for 30 minutes. 100% = 7 units/mg cdw Cyanidase.
G: glucose, F: fructose, S: sucrose, M: maltose, MD: maltodextrin, T: trehalose; PB: 50 mM phosphate buffer; 0.25/0.5: concentration of sugars (molar); 9%/18%: concentration of MD (w/v percentage)

3.2.1.4 ACC deaminase

All the sugars showed protection ability on ACC deaminase at 4°C (Fig 16-A). Cells with 18% maltodextrin showed slightly higher ACC deaminase activity than PB at 15°C, while cells with 0.25 M trehalose or PB maintained highest ACC deaminase activity (Fig 16-B). At 55°C, ACC deaminase of cells with PB or 0.25 M glucose, or 9% maltodextrin was most stable. ACC deaminase was not as stable as other enzymes at 4°C and 15°C (Fig 16-D).

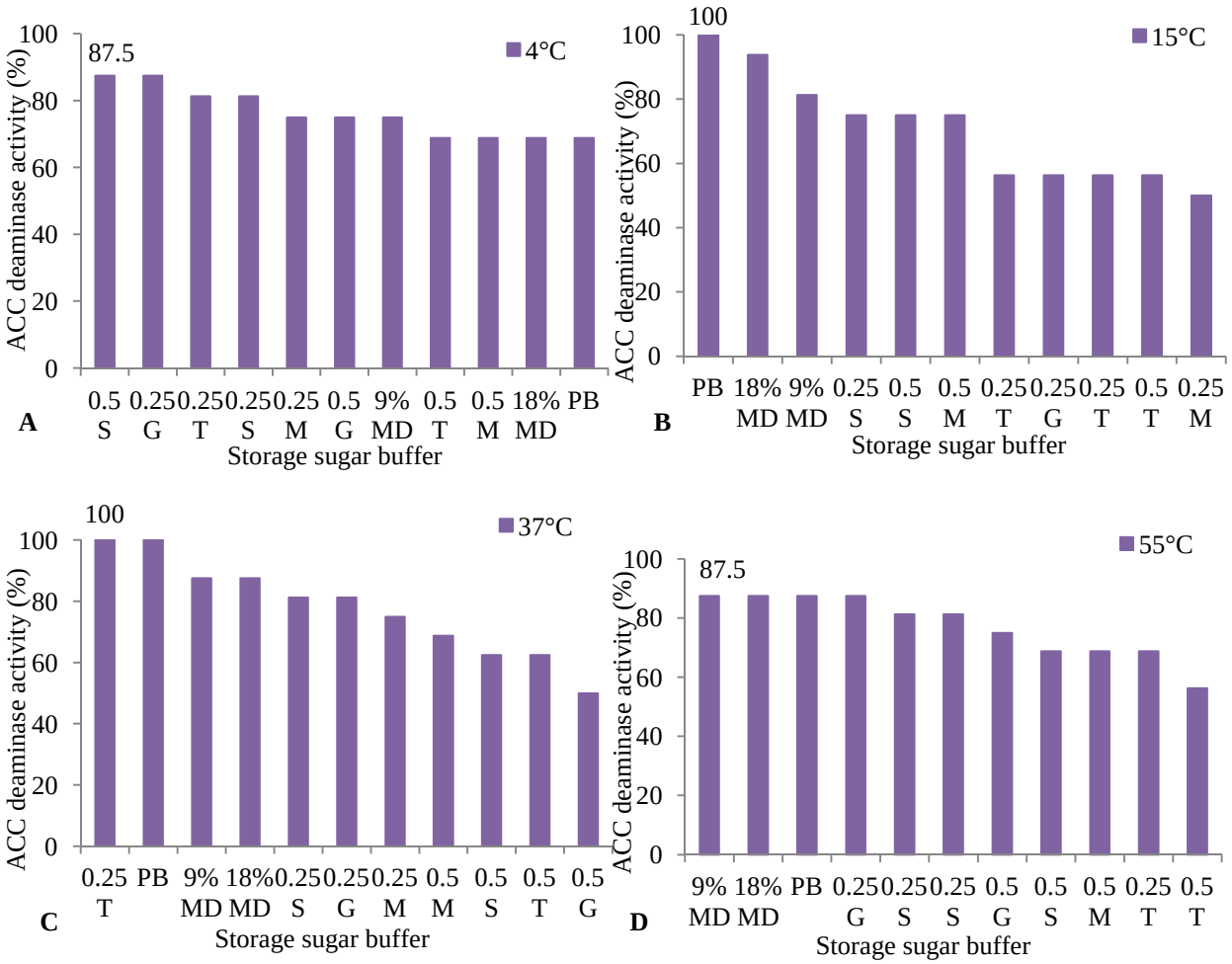


Figure 16. ACC deaminase thermostability in *R. rhodococcus* DAP 96253 whole cells with different sugar buffer incubated at various temperatures for 30 minutes.
100% = 12 units/mg cdw ACC deaminase.
G: glucose, F: fructose, S: sucrose, M: maltose, MD: maltodextrin, T: trehalose; PB: 50mM phosphate buffer; 0.25/0.5: concentration of sugars (molar); 9%/18%: concentration of MD (w/v percentage)

3.2.1.5 βCAS-like Enzyme

βCAS-like enzyme in whole cells was most stable at all the four temperatures tested in this study, almost no loss. Cells with trehalose and maltose could maintain slightly higher βCAS-like activity than other sugar buffers or PB, but it was not significant at 4°C and 15°C. At comparable high temperature, 37°C and 55°C, trehalose showed highest βCAS-like activity, which was significant. Maltose also showed comparable high βCAS-like activity, but it was not significant (Table 10). βCAS-like enzyme was most stable with trehalose incubated at various temperatures for half an hour.

Table 10. Thermostability of βCAS-like activity in *R. rhodococcus* DAP 96253 whole cells with different sugar buffer incubated at various temperatures for 30 minutes

βCAS-like (units/mg cdw)	4°C	15°C	37°C	55°C
0.25 T	9	11	10*	12*
0.50 T	10	10	12*	11*
0.25 M	9	8	7	7
0.50 M	5	5	8	8
0.25 S	6	6	6	5
0.50 S	6	6	6	5
0.25 G	5	6	5	5
0.50 G	5	6	5	5
9% MD	5	5	5	5
18% MD	5	5	5	4
PB	6	6	6	5

Cells were suspended with different sugar buffers, and then incubated at various temperatures for half an hour before testing enzyme activity. G: glucose, F: fructose, S: sucrose, M: maltose, MD: maltodextrin, T: trehalose; PB: 50mM phosphate buffer; 0.25/0.5: concentration of sugars (molar); 9%/18%: concentration of MD (w/v percentage); * statistical significance

3.2.2 Thermostability of Enzymes in Rhodococcus rhodochrous 96622

Comparing to *R. rhodochrous* DAP 96253, NHase in *R. rhodochrous* DAP 96622 was less stable at 4°C and 55°C, whereas amidase was more stable based on the trend lines shown in figure 17-A, B. Cyanidase in *R. rhodochrous* DAP 96253 was slightly less stable at 4°C and 55°C, while cyanidase level in *R. rhodochrous* DAP 96622 was not affected by incubating at

various temperatures for 30 minutes (Fig 17-C). The trend lines of ACC deaminase were similar between the two strains, incubated for half an hour at higher temperature like 37°C and 55°C, this enzyme showed slightly higher activity than lower temperature 4°C, 15°C and RT (Table 11; Fig 17-D). *R. rhodochrous* 96622 showed highest NHase and amidase activity at 37°C, while *R. rhodochrous* 96253 showed highest NHase and amidase activity at RT (Fig 17). Both *R. rhodochrous* 96622 and *R. rhodochrous* 96253 showed highest βCAS-like activity at RT (Fig 17-E).

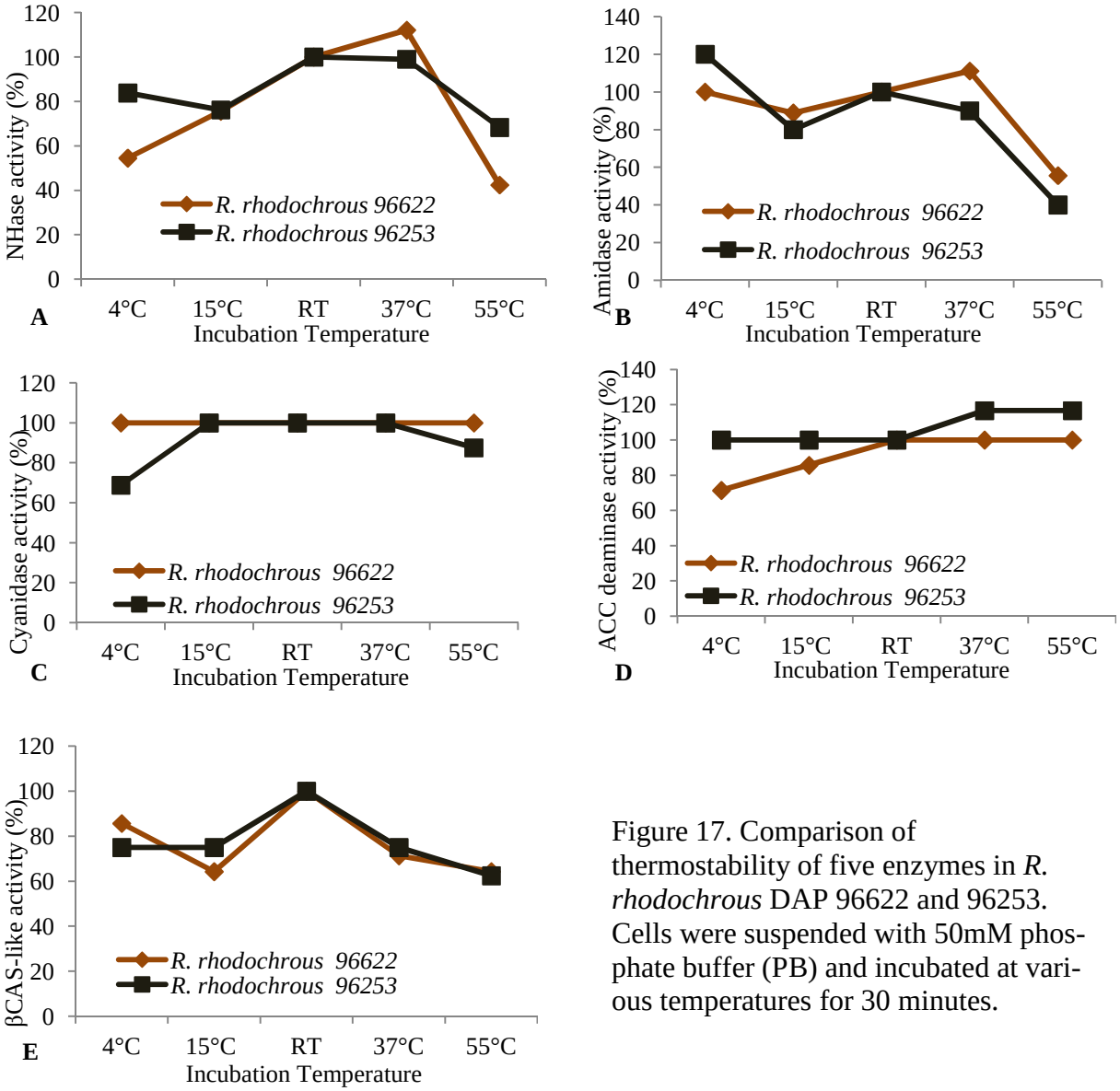


Figure 17. Comparison of thermostability of five enzymes in *R. rhodochrous* DAP 96622 and 96253. Cells were suspended with 50mM phosphate buffer (PB) and incubated at various temperatures for 30 minutes.

Table 11. Enzyme activity relative to 100% as shown in Figure 17 for *R. rhodochrous* DAP 96253 and *R. rhodochrous* DAP 96622

	NHase (units/mg cdw)	Amidase (units/mg cdw)	Cyanidase (units/mg cdw)	ACC deaminase (units/mg cdw)	βCAS-like enzyme (units/mg cdw)
R. rhodochrous DAP 96253 (100%)	210	21	7	18	8
R. rhodochrous DAP 96622 (100%)	40	9	7	7	14

3.2.3 Effects of Different Storage Buffer on Stability of Enzymes of Fermented R. rhodochrous DAP 96253 cells at -20°C, 4°C, and Room Temperature

After storage for 7 days, cells in PB at -20°C showed the highest NHase activity and cells in M9 at 4°C showed the highest amidase activity, while in Asparagine (Asn) at RT showed the highest cyanidase activity. ACC deaminase was the most unstable and lost most of its activity. βCAS-like enzyme was the most stable among these five enzymes (Table 12).

After 18 days of storage, cells stored with M9 at -20°C, PB at -20°C, and trehalose at 4°C had the highest NHase activity. Amidase was unstable at RT, but it could maintain most of its activity at -20°C and 4°C. βCAS-like enzyme was most stable at -20°C. There were no significant differences among various storage conditions for cyanidase (Table 12).

After storage for 30 days, cells with M9 at 4°C and trehalose at -20°C had the highest NHase activity, amidase and cyanidase lost their activities, and βCAS-like enzyme still maintained most of its activity (Table 12).

After 60 days storage, cells with PB at 4°C and trehalose at -20°C had the highest NHase activity. Amidase stored at -20°C was most stable than at RT and 4°C. βCAS-like enzyme stored with trehalose at 4°C still maintained most of its activity. Cyanidase was most stable when stored at -20°C (Table 12).

Table 12. Enzyme activity of fermented *R. rhodochrous* DAP 96253 stored with different buffer at RT, 4°C, and -20°C after 7 days, 18 days, and 30 days

Buffer and temperature °C	NHase (units/mg cdw)				Amidase (units/mg cdw)				Cyanidase (units/mg cdw)				βCAS-like enzyme (units/mg cdw)			
Storage time (d)	7	18	30	60	7	18	30	60	7	18	30	60	7	18	30	60
M9 RT	232	199	63	6	7	5	6	3	4	3		5	10	6	6	3
M9 4°C	261	254	170	23	17	8	5	5	4	3	2	5	9	5	9	3
M9 -20°C	222	328	43	151	14	11	6	9	2	3	3	8	12	11	7	3
PB RT	236	242		228	4	3	4	3	3	2		3	9	6	5	3
PB 4°C	264	269		265	11	11	5	5	3	3	3	6	9	5	6	3
PB -20 °C	350	331	52	71	7	11	4	9	<1	2	1	5	11	10	8	2
PB+T RT	220	177	121	117	10	3	4	1	3	2		4	8	4	5	3
PB+T 4°C	240	327	114	169	10	11	5	8	3	5	3	6	8	6	9	8
PB+T -20°C	207	306	194	230	9	10	4	8	<1	2	<1	4	8	10	8	4
PB+Asn RT	240	251	97	98	5	5	3	3	5	3		5	12	6	4	4
PB+Asn 4°C	246	286	98	188	10	9	4	6	3	3	3	6	10	6	6	4
PB+Asn -20°C	257	295	45	146	7	10	3	9	3	2	1	4	9	10	8	4

T: 0.5M trehalose; Asn: 1 g/L Asparagine, PB: 50 mM phosphate buffer

3.2.4 Enzyme Stability in Fermented Cells when Stored with M9, Trehalose, and No Buffer (Crude Cell Paste) at Various Temperatures

After 48 hour fermentation, *R. rhodochrous* DAP 96253 cells were harvested and stored in different conditions. After storage for 7 days, cells stored with M9 at 4°C had the highest NHase activity. Amidase maintained most of its activity without buffer at 4°C. Cells with no buffer at RT had the highest ACC deaminase activity and cyanidase activity. There were no significant differences in the various storage conditions on βCAS-like enzyme. This enzyme was stable at all conditions (Fig 18).

After storage for 14 days, cells stored with no buffer at 4°C had the highest NHase activity and amidase activity. Crude cell paste stored with no buffer at RT had the highest cyanidase and ACC deaminase. βCAS-like enzyme remained stable in all conditions (Fig 19).

After storage for 21 days, cells stored with trehalose at RT had the highest NHase activity. Cells stored with M9 at 4°C had the highest amidase activity. Cells stored with no buffer at either 4°C or RT had the highest cyanidase and ACC deaminase activity, but slightly higher βCAS-like enzyme activity than any other condition. βCAS-like enzyme still retained most of its activity (Fig 20).

During the 21 days, βCAS-like enzyme was the most stable. Cell paste at RT could maintain most of its enzyme activity if stored for a short time at both 4°C and RT and after 14 days, there was no loss of ACC deaminase, cyanidase, and βCAS-like enzyme activity in cell paste. However, NHase activity in cell paste was less in cells stored with buffers. PB with trehalose was better for maintaining NHase activity than M9. Amidase in whole cells was more stable at 4°C than RT, and after 14 days, its activity could be better maintained by M9 than PB with trehalose and no buffer.

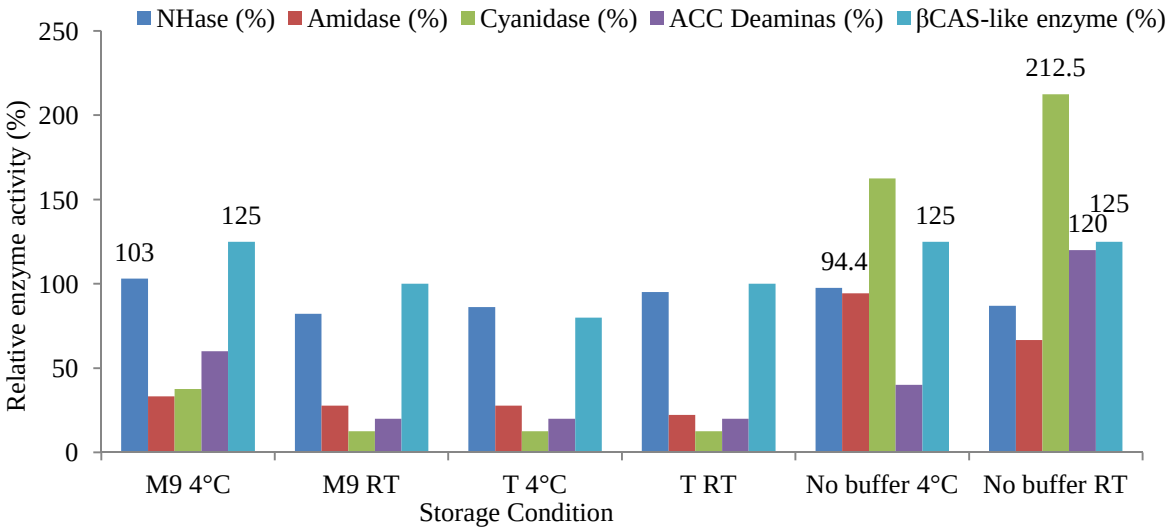


Figure 18. Enzyme activity of fermented *R. rhodochrous* DAP 96253 stored with M9 buffer, 0.25 M trehalose made in 50 mM phosphate buffer (T) and no buffer at RT, and 4°C after 7 days compared to *R. rhodochrous* DAP 96253 cells (CoU) before storage (relative 100%).
100% = 220 units/mg cdw NHase; 29 units/mg cdw Amidase; 12 units/mg cdw ACC deaminase; 7 units/mg cdw Cyanidase; 7 units/mg cdw βCAS-like, separately

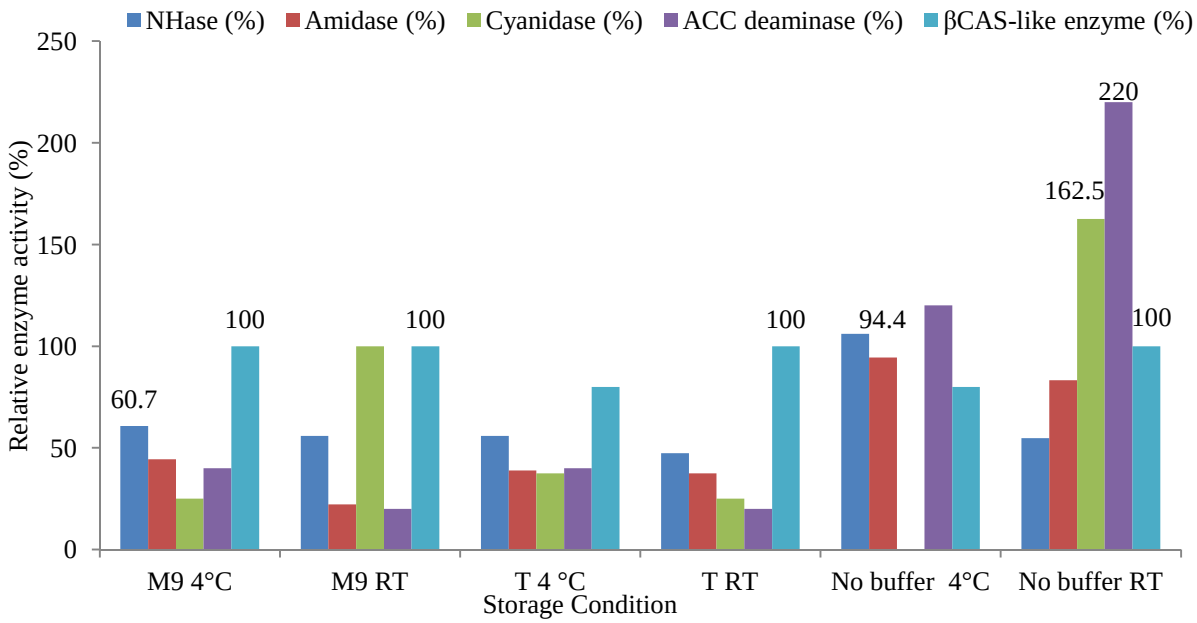


Figure 19. Enzyme activity of fermented *R. rhodochrous* DAP 96253 stored with M9 buffer, 0.25 M trehalose made in 50 mM phosphate buffer (T) and no buffer at RT, and 4°C after 14 days compared to *R. rhodochrous* DAP 96253 cells (CoU) before storage (relative 100%)
100% = 220 units/mg cdw NHase; 29 units/mg cdw Amidase; 12 units/mg cdw ACC deaminase; 7 units/mg cdw Cyanidase; 7 units/mg cdw βCAS-like, separately

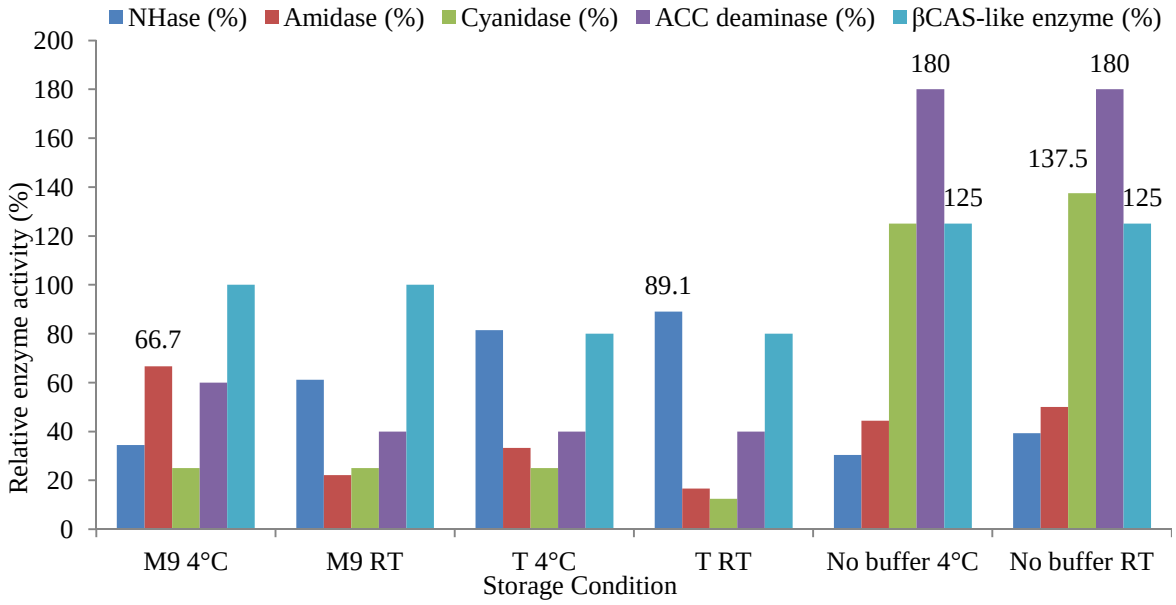


Figure 20. Enzyme activity of fermented cells stored with M9 buffer, 0.25 M Trehalose made in 50 mM phosphate buffer (T), and no buffer, at 4°C and RT after 21 days compared to *R. rhodochrous* DAP 96253 cells (CoU) before storage (relative 100%)
100% = 220 units/mg cdw NHase; 29 units/mg cdw Amidase; 12 units/mg cdw ACC deaminase; 7 units/mg cdw Cyanidase; 7 units/mg cdw βCAS-like, separately

3.2.5 Effects of Different Storage Sugar Buffers on Stability of Enzymes of R. rhodochrous DAP 96253 Whole Cells at 4°C, RT, and Dry Condition for Certain Days

After storage at various temperatures for 35 days (long term storage), effects of different sugars on NHase stability were shown in Figure 21. Maltodextrin at 4°C protected NHase activity the best. 0.5 M sucrose and 9% maltodextrin protected NHase activity the best at RT. 0.25 M trehalose and 0.25 M sucrose showed the highest protection of NHase during desiccation. NHase was most unstable during the slow cell desiccation in long term storage. The higher the concentration of sugar in the buffer, the better it is at protecting NHase activity at 4°C (Fig 21).

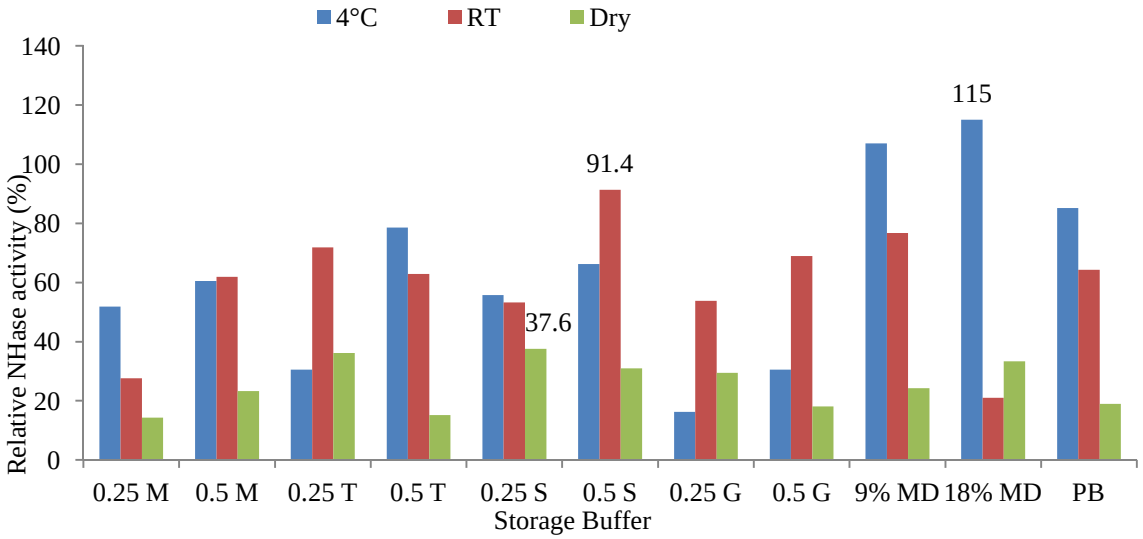


Figure 21. NHase activity of *R. rhodochrous* DAP 96253 cells storage with different sugars under various storage conditions for 35 days
G: glucose, F: fructose, S: sucrose, M: maltose, MD: maltodextrin, T: trehalose; PB: 50 mM phosphate buffer; 0.25/0.5: concentration of sugars (molar); 9%/18%: concentration of MD (w/v percentage); enzymes activities of *R. rhodochrous* DAP 96253 (CoU) before storage (220 units/mg cdw) were used as indicators of 100%, separately

After storage for 35 days, cells with 9% maltodextrin at 4°C showed the highest amidase activity. 18% maltodextrin maintained the highest amidase activity at room temperature. Amidase was most stable with 0.5 M glucose during cell desiccation. Cell desiccation did not affect amidase stability as much as NHase stability (Fig 22).

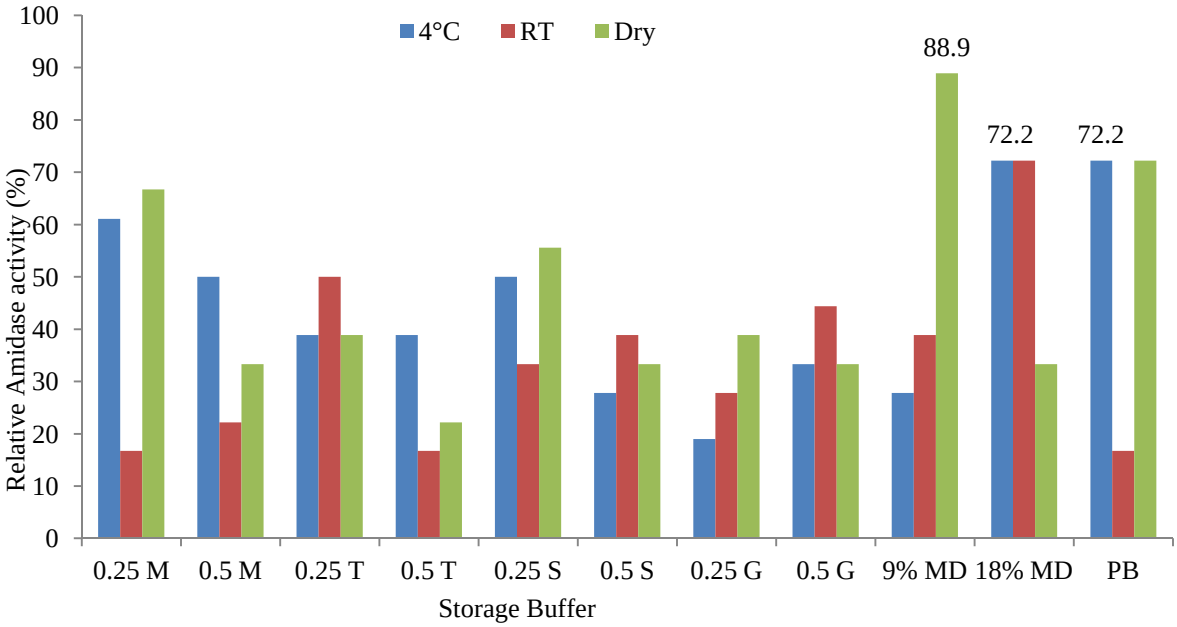


Figure 22. Amidase activity of *R. rhodochrous* DAP 96253 cells stored with different sugars under various storage conditions for 35 days
G: glucose, F: fructose, S: sucrose, M: maltose, MD: maltodextrin, T: trehalose; PB: 50 mM phosphate buffer; 0.25/0.5: concentration of sugars (molar); 9%/18%: concentration of MD (w/v percentage); enzymes activities of *R. rhodochrous* DAP 96253 (CoU) before storage were used as indicators of 100%, separately

3.2.6 Dry cells with Different Sugar Buffer at Different Temperature

3.2.6.1 NHase

After drying in the vacufuge at 30°C with different sugar buffers, cells were stored at various temperatures for 20 days. NHase was the most stable at 15°C with 18% maltodextrin (Fig 23-C). At 4°C, 18% maltodextrin still had the best protective effect on NHase (Fig 23-B). At -20°C, 0.5 M glucose can maintain most of its NHase activity (Fig 23-A). At RT, NHase was most stable with 18% maltodextrin (Fig 23-D). At 55°C, 0.5 M sucrose showed the best protective effect on NHase stability (Fig 23-F). When the storage temperature reached 37°C, cells with the sugars, maltose or glucose, lost NHase activity (Fig 23-E), whereas at -20°C, NHase dried with glucose was the most stable (Fig 23-A).

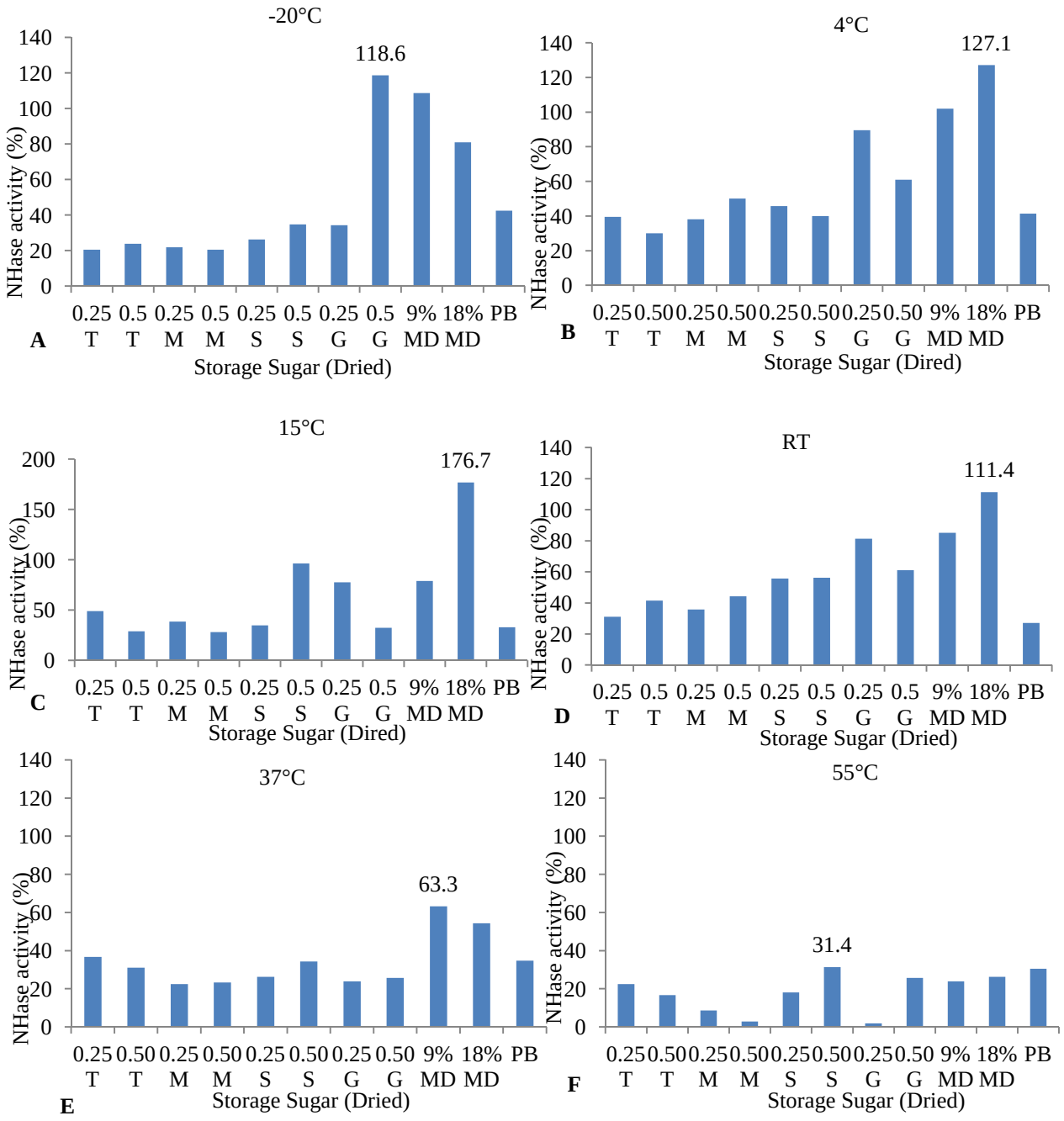


Figure 23. NHase activity of *R. rhodochrous* DAP 96253 cells dried with different sugar buffers and stored at different temperatures for 20 days

G: glucose, F: fructose, S: sucrose, M: maltose, MD: maltodextrin, T: trehalose; PB: 50 mM phosphate buffer; 0.25/0.5: concentration of sugars (molar); 9%/18%: concentration of MD (w/v percentage); enzymes activities of *R. rhodochrous* DAP 96253 (CoU) before vacufuge dried (220 units/mg cdw) were used as indicators of 100%, separately

3.2.6.2 Amidase

After 20 days incubation at various temperatures, maltodextrin did not show similar protection of amidase as NHase, except for -20°C (Fig 24-A). Amidase in whole cells dried with trehalose was most stable at -20°C, 4°C, RT, 37°C and 55°C, while at 15°C, amidase in whole cells dried with maltose was the most stable. With storage at 4°C and RT, there was similar protection by sugars on the amidase activity in dried whole cells, while at 15°C, the amidase in whole cells dried with various sugar buffers showed different activity comparing to 4°C and 15°C (Fig 24-B, C, D). Amidase in whole cells dried without sugar (PB) lost most of its activity when storage at 37°C and 55°C (Fig 24-E, F), while NHase in whole cells dried without sugar still had some activity at these two temperatures (Fig 23-E, F). Trehalose and sucrose showed the highest protection for amidase at 55°C, when amidase was most unstable (Fig 24-F).

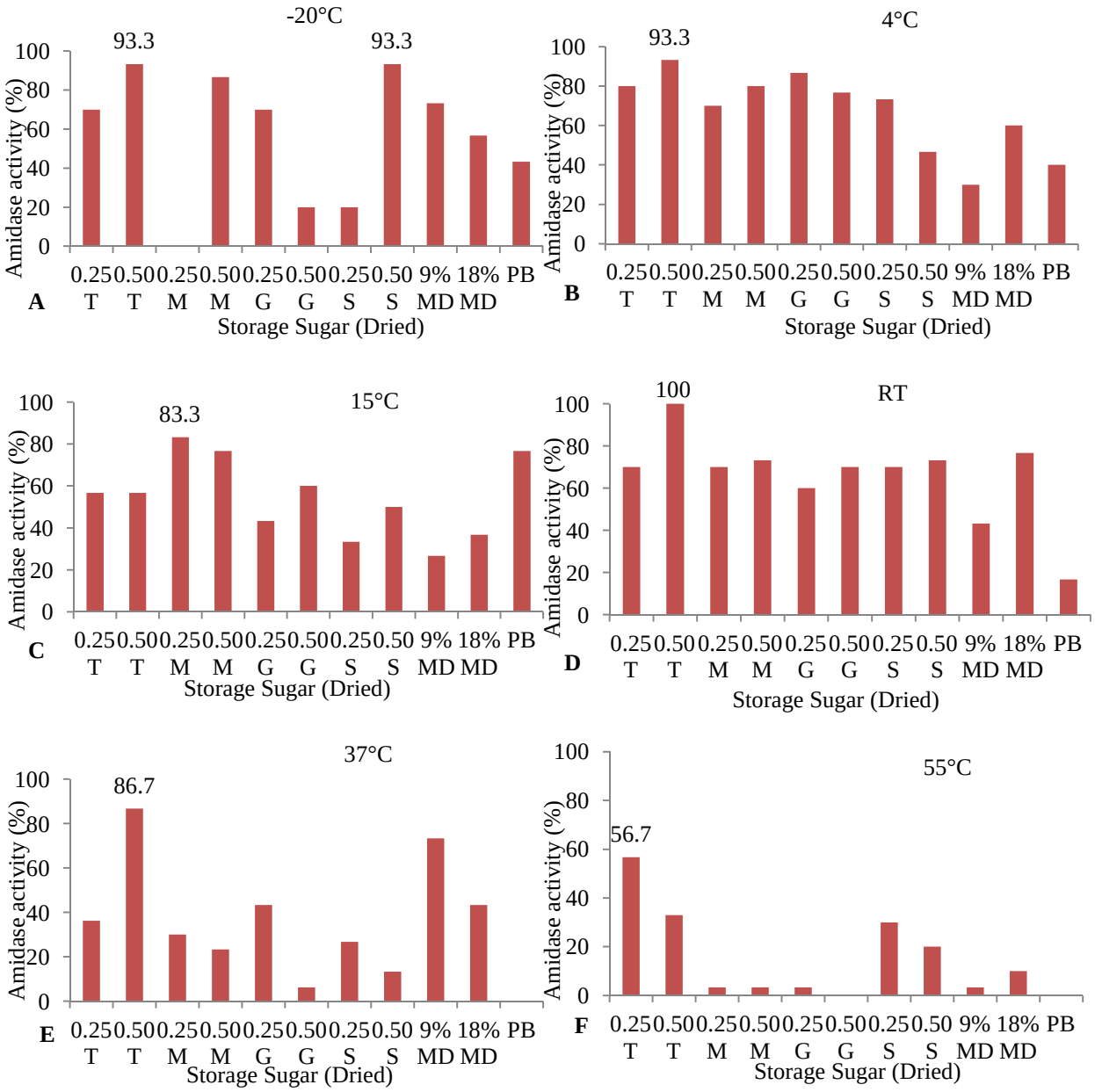


Figure 24. Stability of Amidase from *R. rhodochrous* DAP 96253 whole cells after 20 days stored at various temperatures

G: glucose, F: fructose, S: sucrose, M: maltose, MD: maltodextrin, T: trehalose; PB: 50 mM phosphate buffer; 0.25/0.5: concentration of sugars (molar); 9%/18%: concentration of MD (w/v percentage); enzymes activities of *R. rhodochrous* DAP 96253 (CoU) before vacufuge dried (29 units/mg cdw) were used as indicators of 100%, separately

3.2.6.3 Cyanidase

Cyanidase dried with maltodextrin was most stable at 4°C, 15°C, RT, 37°C and 55°C (Fig 25). At -20°C, cyanidase dried with PB was the most stable (Fig 25-A). Similar to amidase, at 55°C, trehalose and sucrose showed the highest protection for cyanidase while maltodextrin at this temperature showed minor protective ability (Fig 25-F), which was similar to amidase. Cyanidase in whole cells dried without sugar (PB) lost most of its activity when storage was at 37°C and 55°C (Fig 25-E, F), which was similar to amidase (Fig 25-E, F). Maltose, especially in high concentrations, 0.5 M, could maintain 65% of the cyanidase activity, however, when stored at 55°C, cells with maltose lost most of the cyanidase activity (Fig 25-F). Cyanidase in whole cells dried with trehalose were less stable at -20°C and 4°C than at higher temperatures 15°C, RT, 37°C and 55°C (Fig 25).

3.2.6.4 ACC deaminase

ACC deaminase was not as stable as other enzymes when stored at -20°C (Fig 26-A). After 20 days stored at -20°C, whole cells dried with sugars did not have as high ACC deaminase activity as dried without sugar (PB) (Fig 26-A). ACC deaminase dried with maltodextrin was most stable at -4°C, 15°C and RT (Fig 26-B, C, D). Effects of sugars on ACC deaminase, amidase, and cyanidase were similar at 37°C and 55°C. At 55°C, trehalose and sucrose showed the highest protection for ACC deaminase while maltodextrin had slight protective ability at this high temperature (Fig 26-F). At 37°C, cells with maltose also maintained 35% ACC deaminase activity, however, at 55°C, most of the enzyme activity was lost (Fig 26-E).

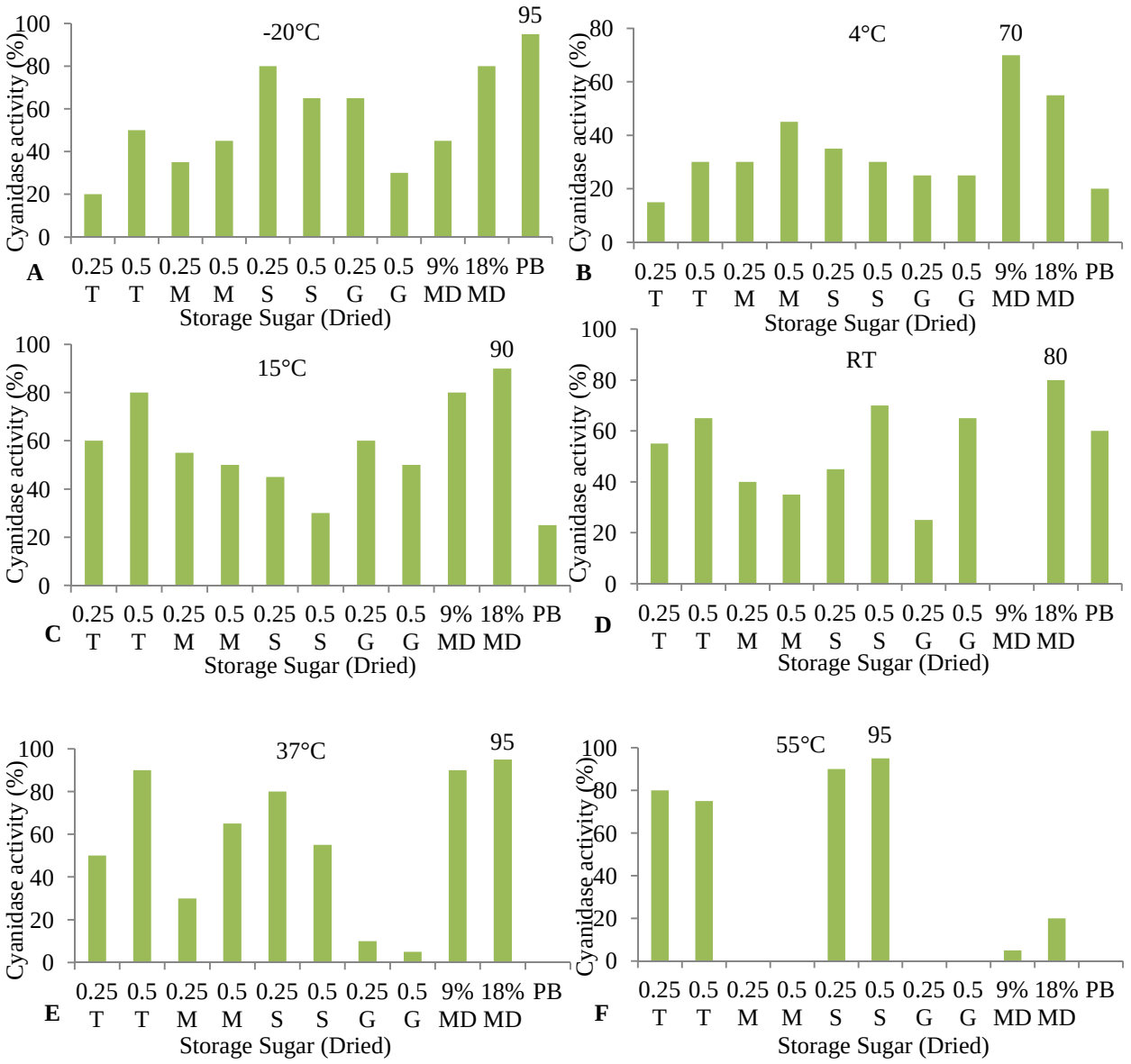


Figure 25. Cyanidase activity of *R. rhodochrous* DAP 96253 cells dried with different sugar buffers and stored at different temperatures for 20 days

G: glucose, F: fructose, S: sucrose, M: maltose, MD: maltodextrin, T: trehalose; PB: 50 mM phosphate buffer; 0.25/0.5: concentration of sugars (molar); 9%/18%: concentration of MD (w/v percentage); enzymes activities of *R. rhodochrous* DAP 96253 (CoU) before vacufuge dried (7 units/mg cdw) were used as indicators of 100%, separately

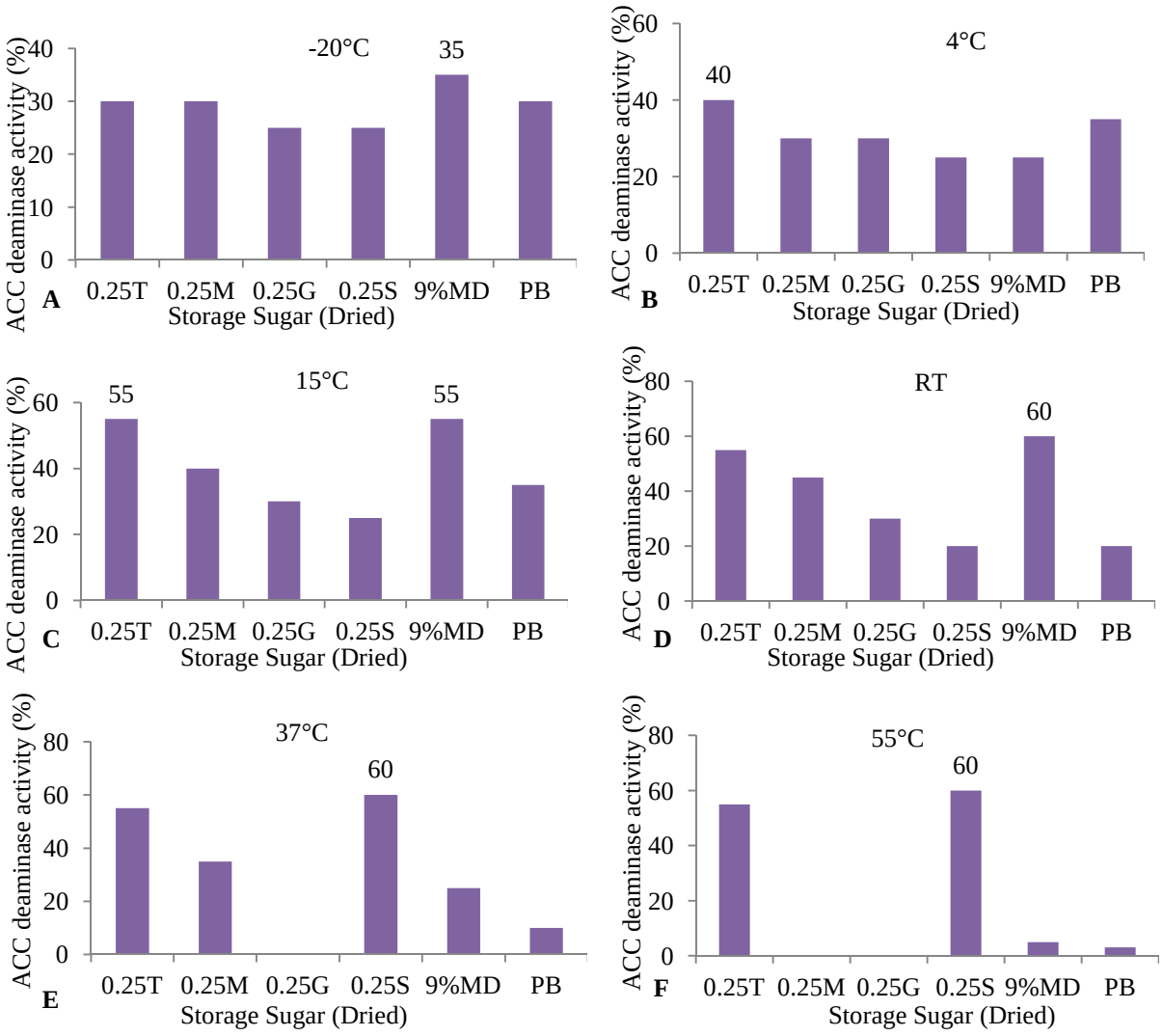


Figure 26. βCAS-like activity of *R. rhodochrous* DAP 96253 cells dried with different sugar buffers and stored at different temperatures for 20 days
G: glucose, F: fructose, S: sucrose, M: maltose, MD: maltodextrin, T: trehalose; PB: 50 mM phosphate buffer; 0.25/0.5: concentration of sugars (molar); 9%/18%: concentration of MD (w/v percentage); enzymes activities of *R. rhodochrous* DAP 96253 (CoU) before vacufuge dried (12 units/mg cdw) were used as indicators of 100%, separately

3.2.6.5 βCAS-like

Different from other enzymes, βCAS-like enzyme was stable at 55°C. The loss of this enzyme at various temperatures in vacufuge-dried cells was not significant based on the statistical analysis. At -20°C, RT, and 37°C, βCAS-like enzyme dried with trehalose was the most sta-

ble. At 4°C and 15°C, βCAS-like enzyme dried with PB was most the stable. At 55°C, βCAS-like enzyme in dried *R. rhodochrous* DAP 96253 cells was the most stable among the five enzymes tested (Table 13).

Table 13. βCAS-like activity of *R. rhodochrous* DAP 96253 cells dried with different sugar buffers and stored at different temperatures for 20 days

βCAS-like (units/mg cdw)	-20°C	4°C	15°C	RT	37°C	55°C
0.25 T	8	8	9	9	7	7
0.5 T	9	9	10	10	8	6
0.25 M	6	5	6	6	5	8
0.5 M	5	4	6	5	4	9
0.25 G	7	6	8	6	5	8
0.5 G	6	6	7	5	6	9
0.25 S	7	6	7	8	7	6
0.5 S	8	8	8	9	7	7
9% MD	7	7	5	8	6	7
18% MD	6	6	4	7	6	6
PB	8	9	11	9	7	6

Cells were suspended with different sugar buffers, dried at 30°C by vacufuge for 7 hours, and then stored at various temperatures for 20 days.

3.2.7 Comparison of Different Immobilization Methods on Enzyme Stability of Rhodococcus rhodochrous DAP 96253

Differing effects on enzyme stability by different immobilization methods are shown in Figure 28. Both carnauba wax and glutaraldehyde immobilization had better effects on maintaining enzyme activity. These two methods maintained approximately 25% of NHase activity and βCAS-like enzyme activity. None of the three immobilization methods had significant protection on amidase, cyanidase, and ACC deaminase (Fig 27; Fig 28). Lyophilized *R. rhodochrous* DAP 96253 cells also maintained 25% of NHase activity. However, at the meantime, the lyophilized cells maintained 68.4% of amidase activity and 41.7% of cyanidase activity, which was more than immobilized cells.

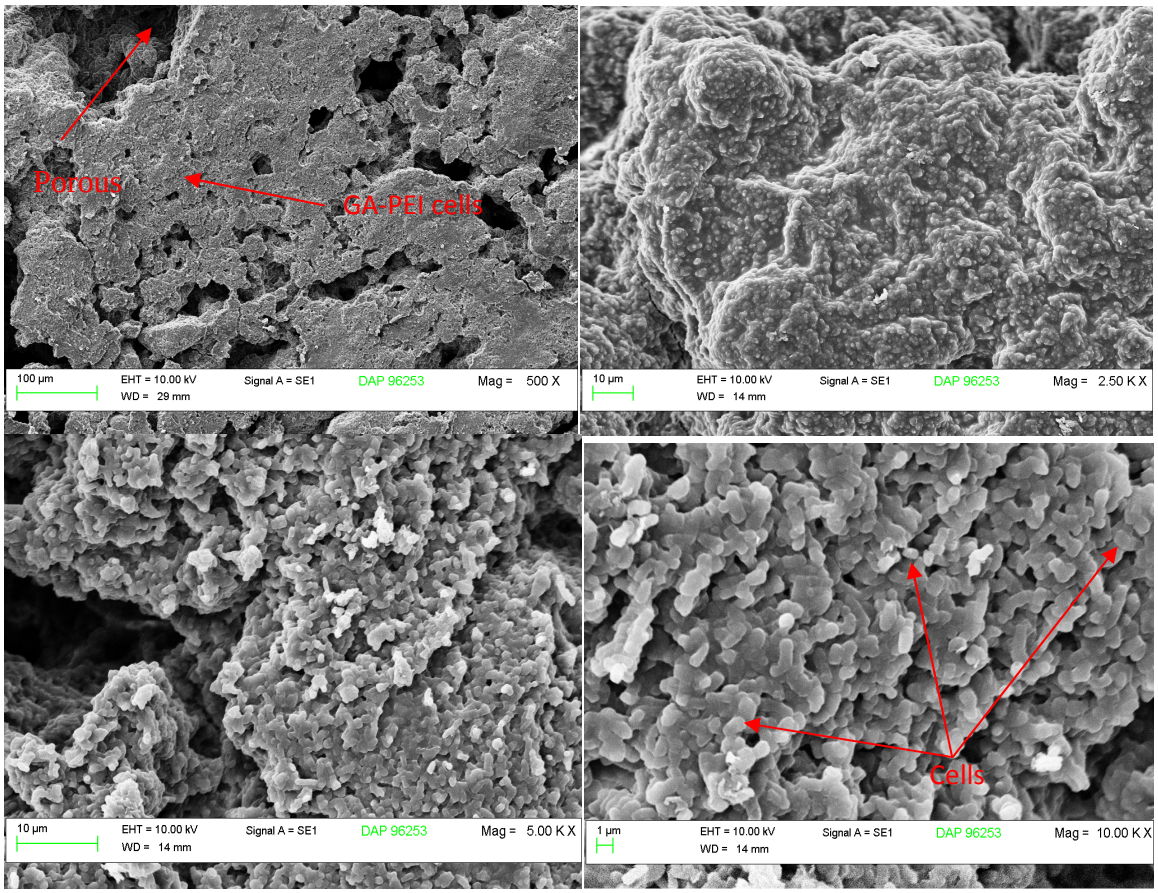


Figure 27. SEM photos of GA-PEI immobilized *R. rhodochrous* DAP 96253 cells (scraped from YEMEA with CoU plates). All SEM images performed by Dr. Robert B. Simmons.

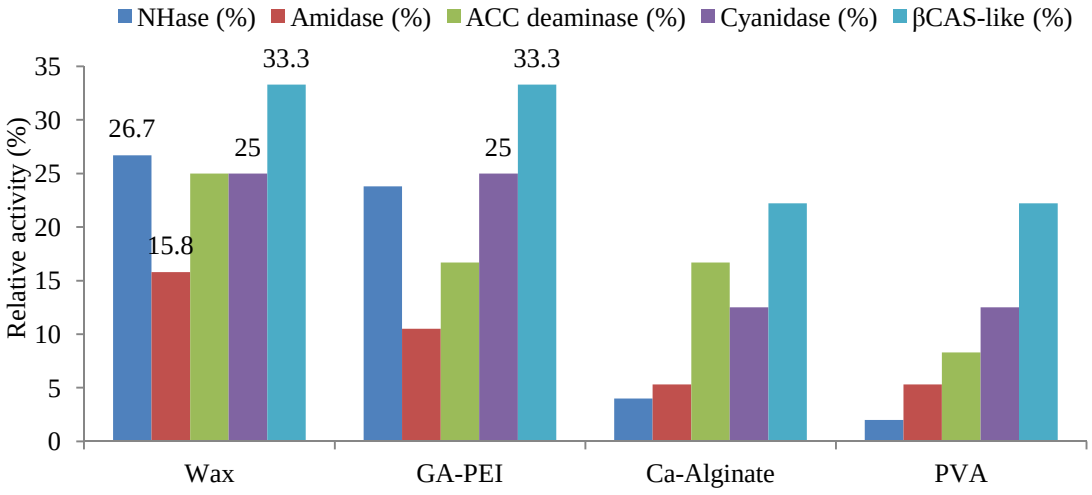


Figure 28. Effect of wax, glutaraldehyde (GP), CA-alginate (Beads) immobilization on enzyme activity. Cells prior to immobilization=100% = 220 units/mg cdw NHase; 29 units/mg cdw Amidase; 12 units/mg cdw ACC deaminase; 7 units/mg cdw Cyanidase; 7 units/mg cdw βCAS-like, separately. Wax: carnauba wax immobilized cells; GA-PEI: glutaraldehyde immobilized cells; Ca-Alginate: Calcium-alginate immobilized cells; PVA: cells immobilized on PVA (Lentikat) (commercially provided)

3.3 Fruit Ripening

3.3.1 Effects of R. rhodochrous DAP 96253 Cells from YEMEA with Various Inducers on Bananas Ripening

Photos were taken after six days. They showed that *R. rhodochrous* DAP 96253 cells scraped from various YEMEA plates could delay fruit ripening, even from YEMEA plates without additional inducers. Out of the various inducers tested, cells grown with cobalt did not show a significant effect on delayed banana ripening. Bananas with cells from YEMEA plates with urea or urea and cobalt showed delayed ripening (Fig 29).

Figure 29. Bananas storage with *R. rhodochrous* DAP 96253 scraped from different YEMEA plates after six days.
a. Control; b. Bananas storage with *R. rhodochrous* DAP 96253 scraped from uninduced plates
c. Bananas storage with *R. rhodochrous* DAP 96253 scraped from GCoU
d. Bananas storage with *R. rhodochrous* DAP 96253 scraped from GU
e. Bananas storage with *R. rhodochrous* DAP 96253 scraped from GCo

3.3.2 Effects of live and Immobilized R. rhodochrous DAP 96253 Cells from CoU on Bananas Ripening

Photos were taken after four days and seven days (Fig 30). At day four, the control bananas showed ripening as indicated by multiple black spots shown on the peels of bananas (Fig 30-A.a). However, the test bananas with catalysts, both live and immobilized *R. rhodochrous* DAP 96253 cells from CoU plates, were still in good condition and there was no indication of any black spots on the peels (Fig 30-B.a, C.a). The control bananas with spinach also showed ripening, with a few black spots on the peels, but this result was better than just bananas alone (Fig 30-A.a, D.a). The test bananas with spinach and catalysts were in good condition without any black spots on the peels (Fig 30-E.a, F.a). The bananas with spinach and live catalysts sealed in dried paper bags had a faster ripening rate than bananas sealed in plastic boxes. The differences between the control groups and test groups were more significant (Fig 31). Both the live and dead catalysts reduced the ripening of the bananas, and spinach also had some effect on the ripening of the bananas.

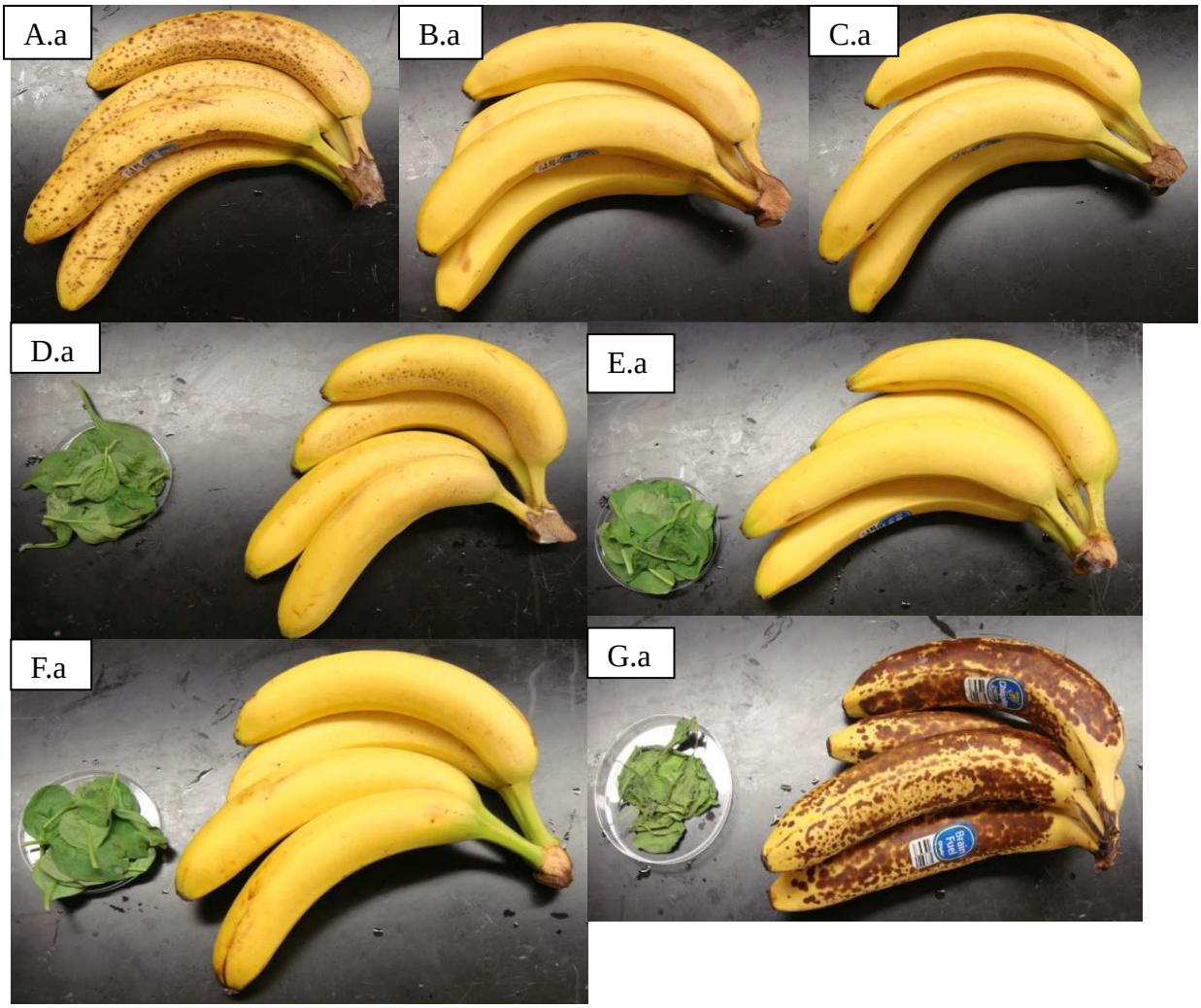


Figure 30. A.a-G.a: Bananas ripening experiment after four days
A.a Bananas control; B.a Bananas + fresh cells; C.a Bananas + GP immobilized cells; D.a Bananas + Spinach; E.a Bananas + spinach + fresh cells; F.a Bananas + Spinach + GP immobilized cells; G.a Bananas + Spinach + fresh cells (sealed in paper bag with a beaker of water)

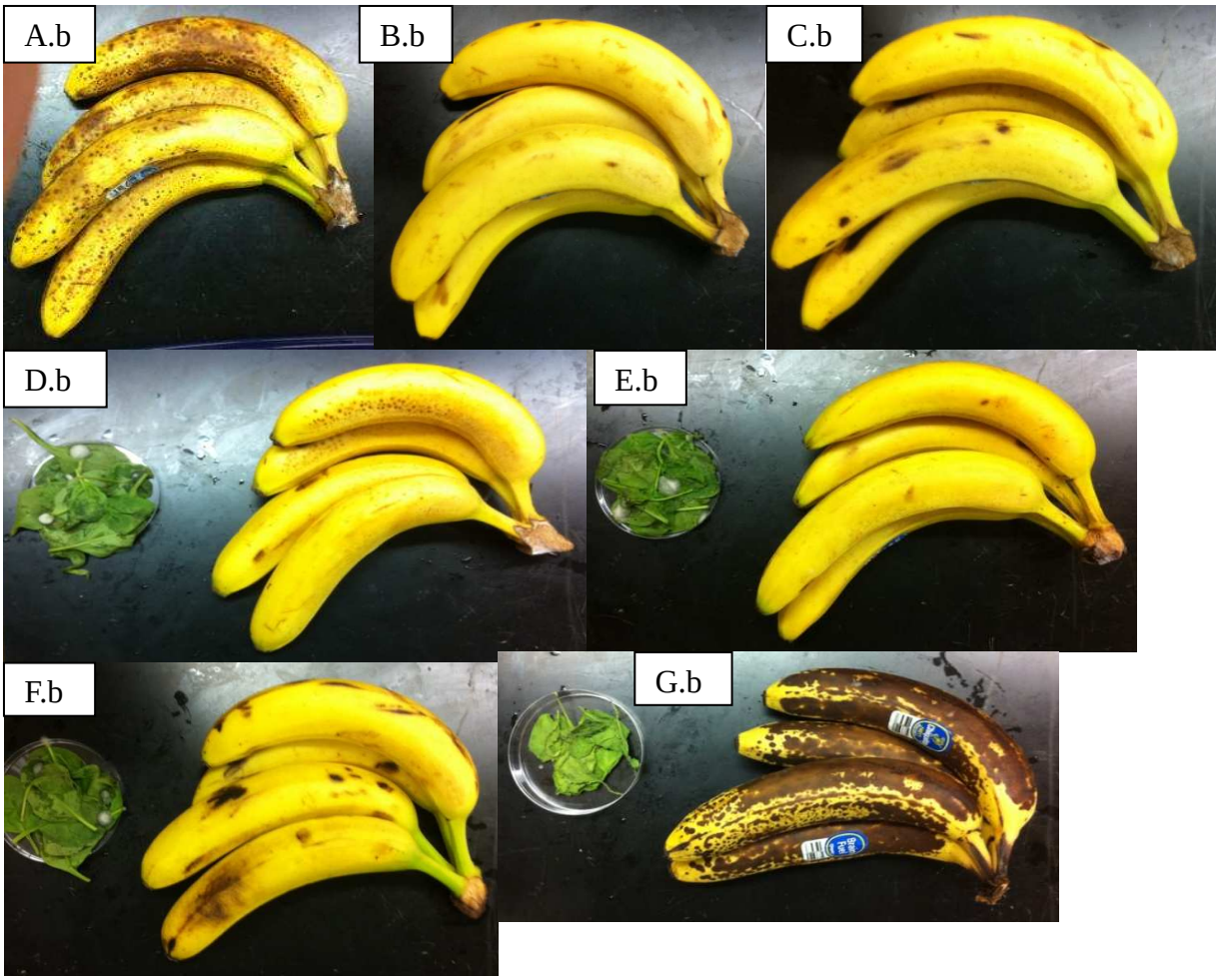


Figure 31. A.b-G.b: Bananas ripening experiment after seven days
A.b Bananas control; B.b Bananas + fresh cells; C.b Bananas + GP immobilized cells; D.b Bananas + Spinach; E.b Bananas + spinach + fresh cells; F.b Bananas + Spinach + GP immobilized cells; G.b Bananas + Spinach + fresh cells (sealed in paper bag with a beaker of water)

3.3.3 Effects of Live and GA-PEI immobilized R. rhodochrous DAP 96253 Cells from Fermentation on Bananas Ripening

After 14 days, bananas placed in different amounts of both live and immobilized *R. rhodochrous* DAP 96253 in sealed 4.4 L plastic boxes showed different ripening effects (Fig 32). The immobilized cells made from 5 g of live fermented cells showed the best effect on delaying the ripening of bananas (Fig 32-F). The control bananas without catalysts or immobilization materials ripened faster than the other groups (Fig 32-A). Compared to control bananas, bananas with immobilization materials (no catalyst) were in an even better condition than bananas with 1

g of fermented cells (Fig 32-A, B, C). Immobilized cells were more effective at delaying fruit ripening than live fermented cells. Bananas placed with more fermented cells, either live or immobilized, showed a much slower rate of ripening (Fig 32-C, D, E, and F).

Figure 32. Bananas placed with certain amount of live and immobilized fermented cells in sealed plastic box for 14 days. A. Control (bananas only); B. Bananas with immobilization materials (no cells); C. Bananas with 1 g live fermented cells; D. Bananas with 5 g live fermented cells; E. Bananas with GA-PEI immobilized cells from 1 g live fermented cells; F. Bananas with GA-PEI immobilized cells from 5 g live fermented cells; cell weights were packed whole cells

Not only did the GA-PEI immobilized *R. rhodochrous* DAP 96253 delay ripening in yellow bananas, it also showed significant delayed ripening of green bananas (Fig 33). After 46 days in sealed plastic containers, control green bananas turned yellow, began ripening, and molded, while green bananas without contact to GA-PEI immobilized cells were still in good condition as indicated by a lighter, yellow color on the peels and less mold on the bananas. However, mold did grow with GA-PEI immobilized cells (Fig 33-B).

Figure 33. Two set of experiments of green bananas in sealed box for 21 and 46 days, 30 days, separetely. A) control; B) with GA-PEI immobilized *R. rhodochrous* DAP 96253 cells

3.3.4 Peaches Contacted with R. rhodochrous DAP 96253 Cells

After 13 days, a wax and cell mixture sprayed directly on peaches, showed the ability to delay the ripening of peaches (Fig 34). Mineral oil wax with cells sprayed on peaches showed the better effects on reducing peach ripening than carnauba wax with cells. Based on the appearance, the peaches sprayed with carnauba wax with cells lost a great portion of water compared with mineral oil wax-cells mixture; the latter peaches also were brighter in color. Wax coatings showed wax build-up and mottled appearance. The wax coating affected the visual appearance of the peaches whereas the mineral oil coating did not.

Figure 34. Effects of different wax and cells mixture on peaches ripening
A. Peaches sprayed with carnauba wax (emulsion made with NaOH) (NA) + *R. rhodochrous* DAP 96253 cells from GCoU; B. Peaches sprayed with mineral oil wax (M) + *R. rhodochrous* DAP 96253 cells from GCoU; C. Control peaches only

3.3.5 Peaches Non-contacted with R. rhodochrous DAP 96253 Cells

After 13 days, the non-contact experiment peaches with *R. rhodochrous* DAP 96253 cells showed delayed ripening compared to the control using either live or immobilized cells. The cells immobilized by various methods also showed reduction in peach ripening and antifungal effects (Fig 35). Wax emulsions without *R. rhodochrous* DAP 96253 cells were also tested with peaches and there were no significant effects observed on delayed fruit ripening and fungi inhibition (data not shown). Calcium-alginate and wax immobilized cells showed more effects than glutaraldehyde immobilized catalyst on delayed peach ripening and fungal inhibition (Table 14).

Table 14. The five enzymes activities in catalysts used for peach ripening experiment and number of fungi-infected peaches

	NHase (%)	AMD (units/mg cdw)	ACCD (units/mg cdw)	Cyanidase (units/mg cdw)	βCAS (units/mg cdw)	Fungi-infected peaches
CWE+cell	29	14	3	3	13	1
CWE*+cell	29	72	4	3	4	2
M+cell	28	41	5	4	6	1
GA-PEI cell	28	21	8	1	6	3
CoU	100	100	6	6	5	1
Ca-alginate	18	17	7	1	8	1
Control	-	-	-	-	-	4

Cell used in this peach ripening experiment was *R. rhodochrous* DAP 96253 scraped from GCoU plates. CWE+cell: carnauba wax (emulsion made with Nh_4OH) + *R. rhodochrous* DAP 96253 cells from GCoU; CWE*+cell: carnauba wax (emulsion made with NaOH) + *R. rhodochrous* DAP 96253 cells from GCoU; MOE: mineral oil wax (M) + *R. rhodochrous* DAP 96253 cells from GCoU; GP: glutaraldehyde-polyethylenimine immobilized *R. rhodochrous* DAP 96253 cells from GCoU; CoU: *R. rhodochrous* DAP 96253 cells from GCoU; Ca-alginate: calcium-alginate immobilized *R. rhodochrous* DAP 96253 cells from GCoU

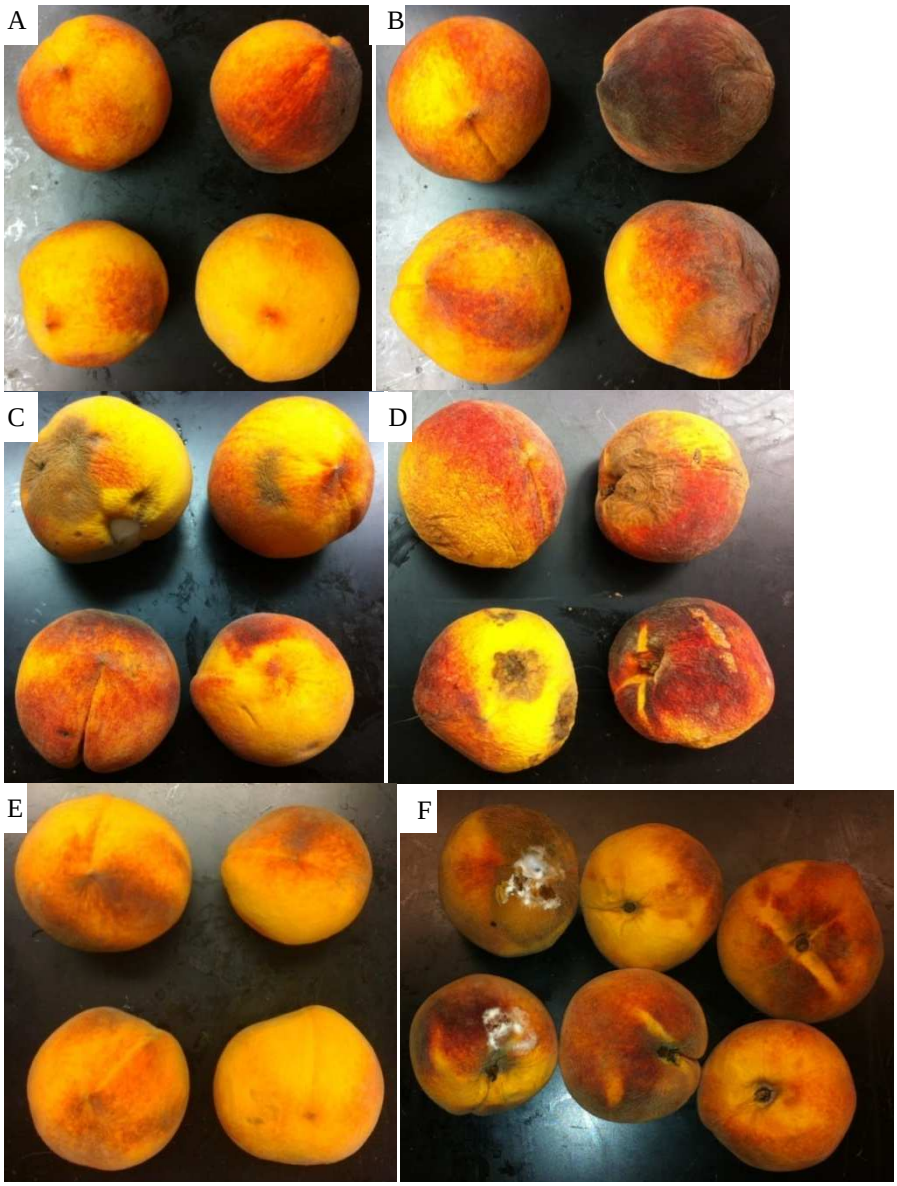


Figure 35**.** Peaches ripening experiment with non-contact wax-cell and calcium-alginate immobilized *R. rhodochrous* DAP 96253 cells from GCoU plates.
A. Sodium alginate beads; B. Live *R. rhodochrous* DAP 96253 cells with M9; C. Mineral oil Wax + *R. rhodochrous* DAP 96253 cells; D. Glutaraldehyde immobilized *R. rhodochrous* DAP 96253 cells; E. CA wax immobilized *R. rhodochrous* DAP 96253 cells; F. control.

3.3.6 Effects of Three Rhodococcus Strains on Bananas Ripening

Bananas sealed with all three *Rhodococcus* strains showed certain delayed fruit ripening. Photos were taken after nine days (Fig 36). *R. rhodochrous* DAP 96622 scraped from YEMEA CoU plates showed a more reddish color than *R. rhodochrous* DAP 96253. After nine days, bananas exposed to *R. rhodochrous* DAP 96622 showed less black spots on the peels than the con-

trol, which indicated that *R. rhodochrous* DAP 96622 cells had the delayed fruit ripening activity. However, this effect was not as significant as *R. rhodochrous* DAP 96253 (Fig 36). Since *R. erythropolis* ATCC 47072 did not grow well on YEMEA CoU plates, the cells used to expose to bananas were scraped from NA plates, which showed a white color. The effect of *R. erythropolis* ATCC 47072 cells on delaying fruit ripening was not significant (data not show).

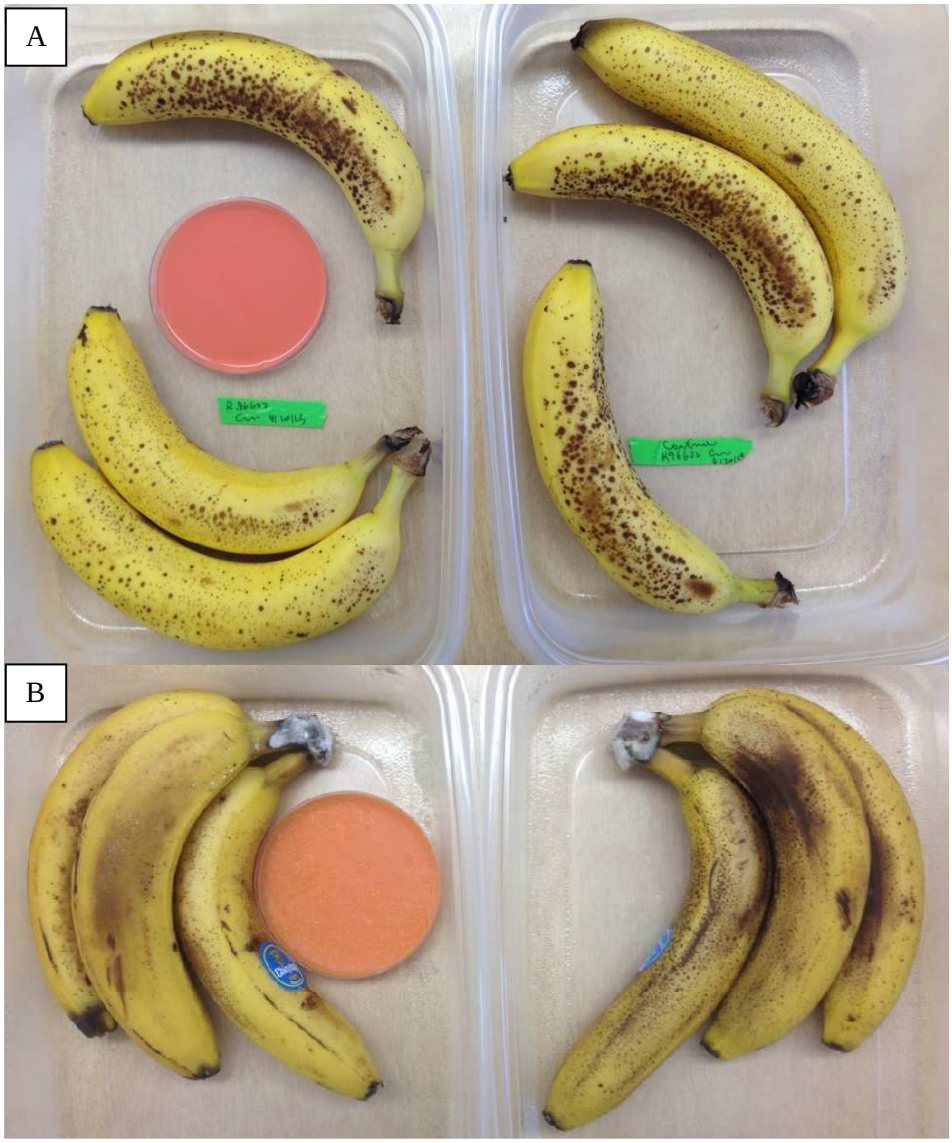


Figure 36. Effects of delaying fruit ripening by *Rhodococcal* strains. A) bananas exposed to *R. rhodochrous* DAP 96622 and control; B) bananas exposed to *R. rhodochrous* DAP 96253

3.3.7 Identify Various Ripening Stages of Bananas

After stained by an iodine solution, the horizontal sections from bananas showed darker to lighter colors indicating their different stages in ripening (Fig 37). A larger majority of ripened bananas, i.e. stage 4, showed a lighter color due to the fact that the starch transformed to sugar during ripening, while the comparable unripe bananas, i.e. stage 1, showed a darker, black color (Fig 37-A, C). The other indicator for banana ripening stages was the peel thickness. Peel thickness of bananas decreased during ripening. On a scale of 1-4, one being the un-ripened banana and four being a ripened banana, the peel thickness of bananas in stage 1 was twice that of stage 4 (Fig 37-A, B).

Figure 37. Peel thickness (B); starch to sugar transformation (C); various stages of banana ripening (A)

3.3.8 Volatiles Produced by Rhodococcus Strains and Cyanide Degradation

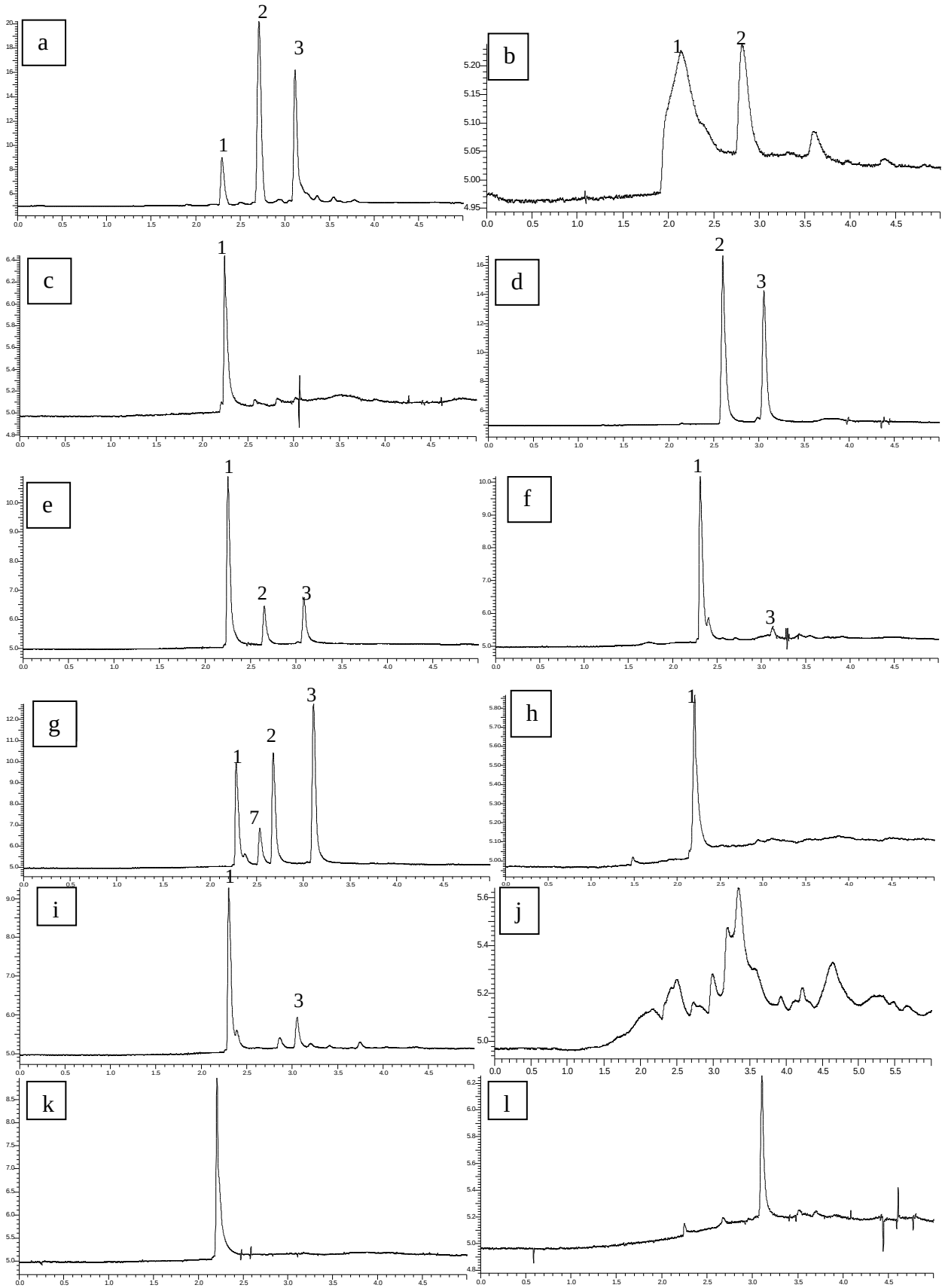
Since all three *Rhodococcus* strains showed delayed banana ripening, their volatile productions were compared (Table 15; Table 16). When comparing *R. rhodochrous* DAP 96253 scraped from YEMEA CoU, NA, and fermentation, the volatile production of cells from YEMEA CoU and fermentation were similar (data not shown), while peak 2 and peak 3 were missing when *R. rhodochrous* DAP 96253 was grown on NA (Fig 38-a, c). *R. erythropolis* ATCC 47072 scraped from NA had two peaks, in which one was similar with *R. rhodochrous* DAP 96253 from CoU or NA that came out at 3.2 minutes (Fig 38-c, d, e). There were few volatiles detected from GP immobilized cells based on the GC method 1, however, which were not similar as *R. rhodochrous* DAP 96253 from CoU (Fig 38-j).

With 5 ml or 15 ml ethylene injection, cells did not show a significant difference in stable degradation of ethylene based on method 1 (data did not show here). With 2 ml KCN (100 ppm), *R. rhodochrous* DAP 96622 and 96253 strains and immobilized *R. rhodochrous* DAP 96253 utilized most of the cyanide, while *R. erythropolis* ATCC 47072 degraded half of the KCN when compared with the control (Fig 38-e, f, g, h, i, k, l).

When comparing bananas with *R. rhodochrous* DAP 96253 cells to bananas via GC method 1, there was a much bigger area count peak that came out at 3.8 minutes, and there was also a peak at 3.2 minutes (Fig 39, Fig 40).

Table 15. Comparison of volatile production among bananas and bananas with *rhodococcal* strains

	Peak 1(2.3)	Peak 2 (2.7)	Peak 3 (3.1)	Peak 4 (2.8)	Peak 5 (3.9)	Peak 6 (4.7)
Bananas (Stage 1)	100%	100%	7.4%	91.5%	16.1%	100%
R. rhodochrous DAP 96253 (CoU) +Bananas	0	97.5%	100%	100%	100%	89.2%
Relative 100%	2441.51	1632.92	17272.01	122669.02	100997.35	6580.80


a
1
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3
b
1
2
c
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d
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3
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Figure 38. Gas headspace results: a. *R. rhodochrous* DAP 96253 cells (from CoU); b. *R. rhodochrous* DAP 96622 cells (from CoU); c. *R. rhodochrous* DAP 96253 cells (from NA); d. *R. erythropolis* ATCC 47072 cells (from NA); e. *R. rhodochrous* DAP 96253 cells (from CoU) with 15 ml ethylene and 2ml KCN; f. *R. rhodochrous* DAP 96253 cells (from NA) with 15 ml ethylene and 2 ml KCN; g. *R. erythropolis* ATCC 47072 cells (from NA) with 15ml ethylene and 2 ml KCN; h. *R. rhodochrous* DAP 96622 (1 g) with 15 ml ethylene and 2 ml 100 ppm KCN; i. GA-PEI immobilized *R. rhodochrous* DAP 96253 (from CoU) catalysts with 15 ml ethylene and KCN; j. GA-PEI immobilized *R. rhodochrous* DAP 96253 (from CoU) catalysts; k. 5ml ethylene (100 ppm); l. 2 ml KCN (100 ppm)

Table 16. Comparison of volatiles production and effects on ethylene and KCN among *rhodococcal* strains

Peak	1	2	3	7
Retention Time (min)	2.3	2.7	3.2	2.5
R. rhodochrous DAP 96253 (CoU)	10856.18	45705.25	35885.74	0
R. rhodochrous DAP 96253 (NA)	3443.91	0	0	0
R. rhodochrous DAP 96622 (CoU)	703.24	193.15	0	0
R. erythropolis ATCC 47072 (NA)	0	34490.17	27813.28	0
GA-PEI *R. rhodochrous* DAP 96253 (CoU)	0	0	0	0
Ethylene	10540.80	0	0	0
KCN	0	0	24497.12	0
R. rhodochrous DAP 96253 (CoU) +Ethylene+KCN	15340.98	3367.28	3893.37	0
R. rhodochrous DAP 96253 (NA) +Ethylene+KCN	13573.81	0	888.57	0
R. rhodochrous DAP 96622+Ethylene+KCN	1077.97	0	0	0
R. erythropolis ATCC 47072+Ethylene+KCN	12803.43	13189.00	19858.84	4387.36
GA-PEI *R. rhodochrous* DAP 96253+Ethylene+KCN	10821.43	0	2053.53	0

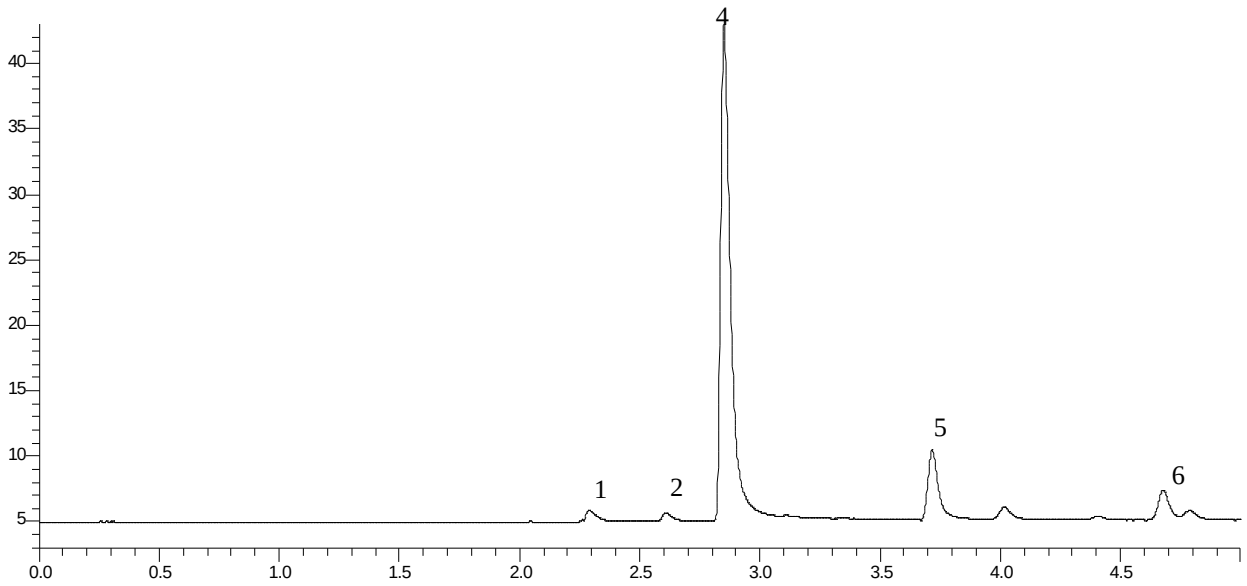

Figure 39. Bananas (Stage 1) only

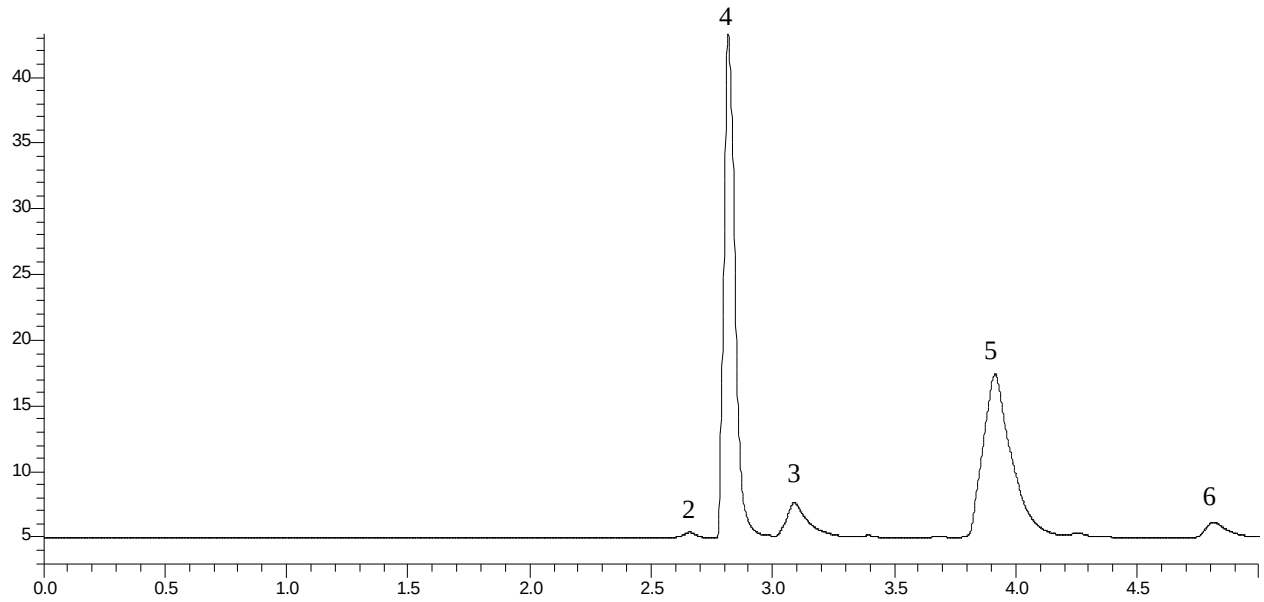

Figure 40. Bananas (Stage 1) with *R. rhodochrous* DAP 96253 cells (CoU)

3.3.9 Volatiles Production from Fruit and Rhodococcus (GC Method 2)

After 48 hours incubation at 30°C with agitation, comparing bananas in various ripening stages, the stage 1 bananas produced less volatile than the stage 2 and 3 bananas (Fig 41; Fig 42; Fig 43). Moreover, there was an increased peak (peak 2) in stage 2 bananas than in stage 1 and 3. Except for the peak 2, stage 3 bananas (late ripening) showed more peaks and larger area

counts than stage 1 bananas, which indicated the aromatic compounds production during ripening (Fig 41; Fig 43). The area counts of peak 2 and 3 from stage 2 bananas were 20% larger than stage 3, while the area counts of peak 5 and 7 from stage 3 bananas were more than 80% larger compared to stage 2 (Table 17).

Compared to stage 2 bananas only, the stage 2 bananas with *R. rhodochrous* DAP 96253 fermented cells have less peaks and area counts (Fig 42; Fig 44; Fig 45). The area counts of peak 1, 2, 4, and 6 of bananas with cells were 71%, 80%, 51%, and 57% less than bananas only, whereas the area counts of peak 7, 8, and 9 were 11%, 12%, and 40% larger than bananas only, separately (Table 17).

Table 17. Peaks and area counts among different stages of bananas (headspace)

Peak	1	2	3	4	5	6	7	8	9
Retention time (min)	~2.183	~2.269	~3.178	~5.822	~5.98	~6.568	~7.099	~8.014	~9.150
BA 1	23%	55%	18%	45%	1%	26%	33%	26%	20%
BA 2	100%	100%	100%	100%	14%	100%	5%	84%	60%
BA 3	79%	0	81%	99%	100%	61%	100%	100%	91%
BA 2 + Fermented cells	29%	20%	95%	49%	11%	43%	16%	96%	100%
Fermented cells	15%	3%	0	0	0	0	0	0	0
Rela-tive100%	153384 .82	305051. 75	45153. 02	25083. 93	22612. 82	53784. 92	26964. 39	11297. 82	23854. 90

BA 1: bananas (stage 1); BA 2: bananas (stage 2); BA 3: bananas (stage 3); Fermented cells: *R. rhodoc*hrous DAP 96253 from fermentation (cells scraped from CoU plates as the inoculum; both cobalt and urea were used as inducers in vessel)

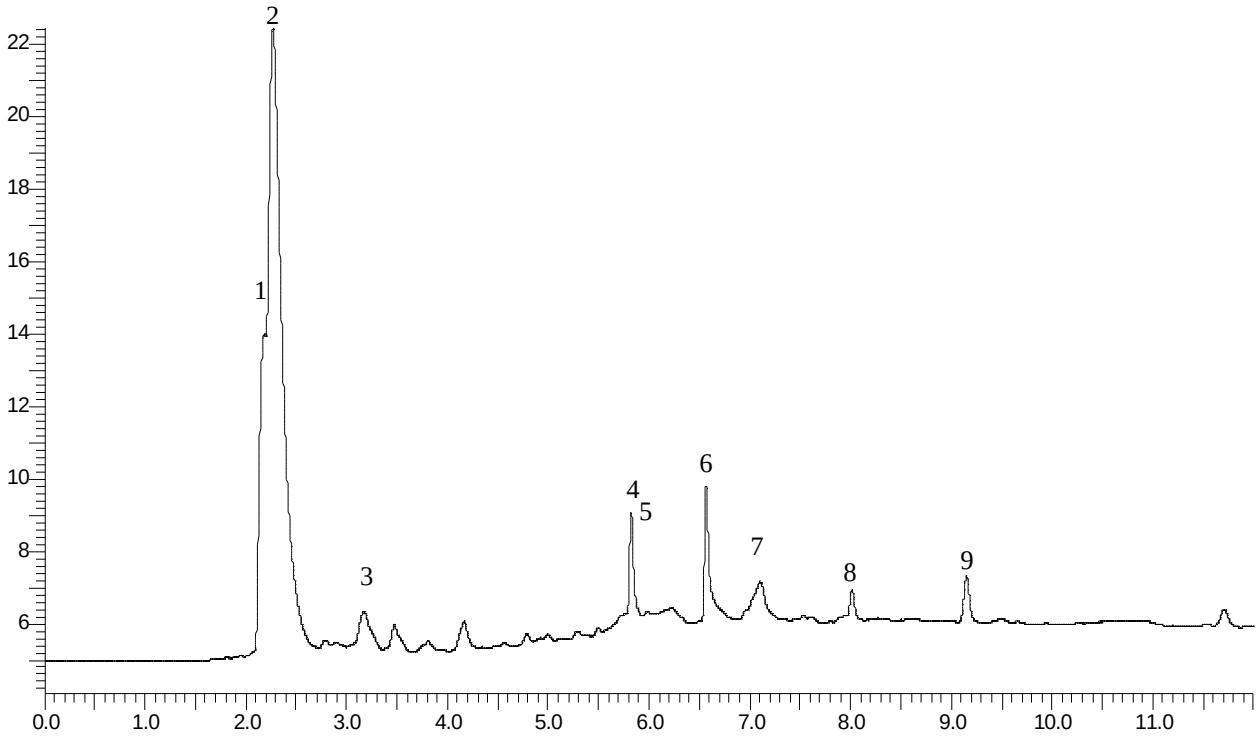

Figure 41. Stage 1 bananas 48 hrs

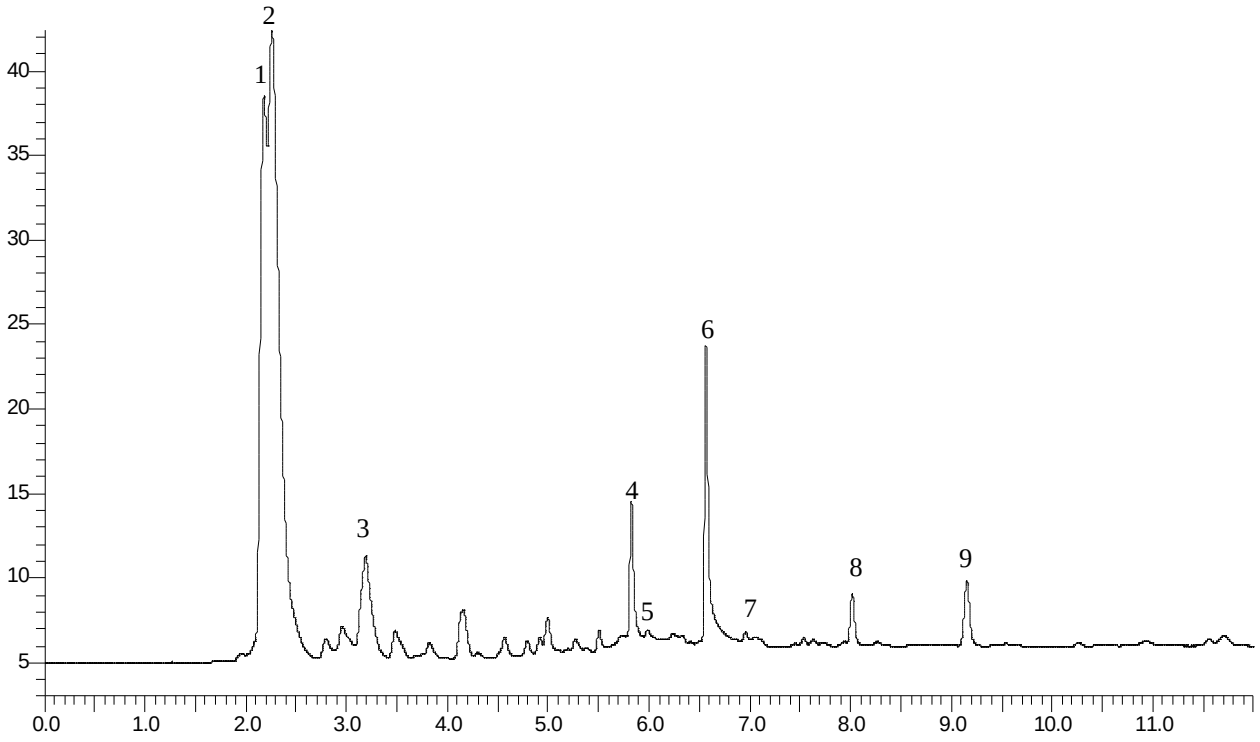

Figure 42. Stage 2 bananas 48 hrs

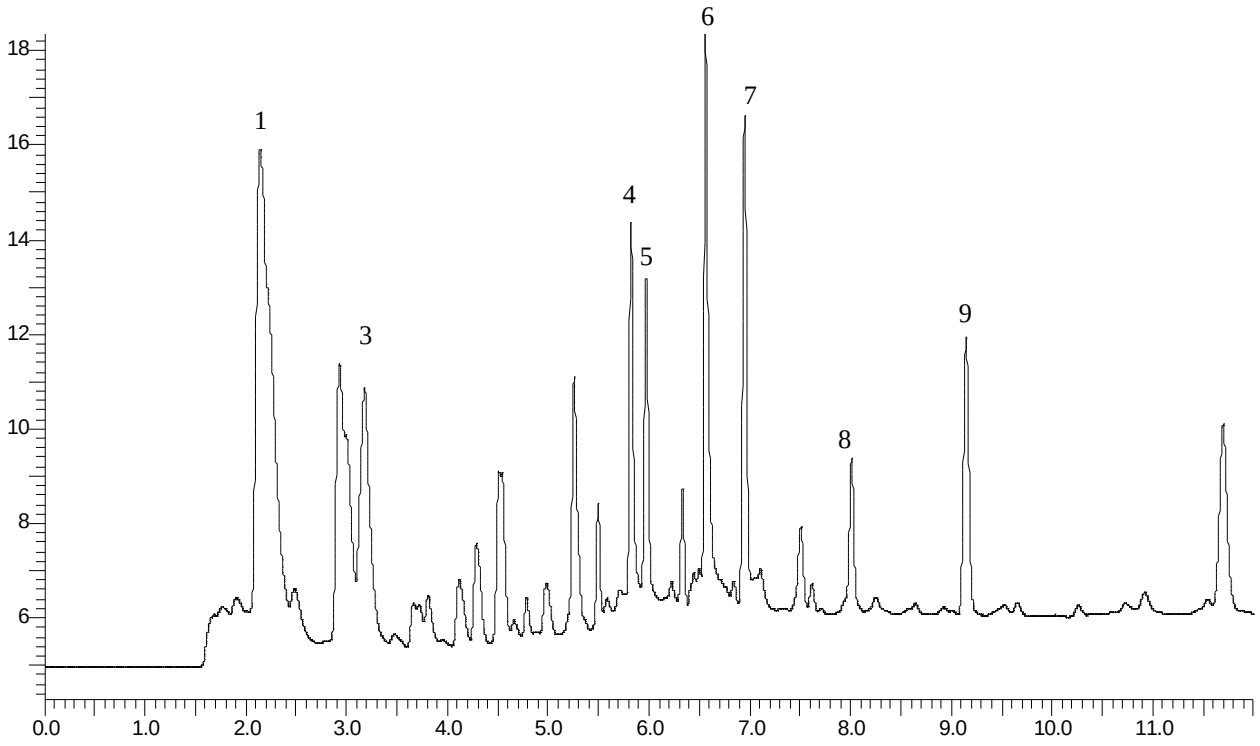

Figure 43. Stage 3 bananas 48 hrs

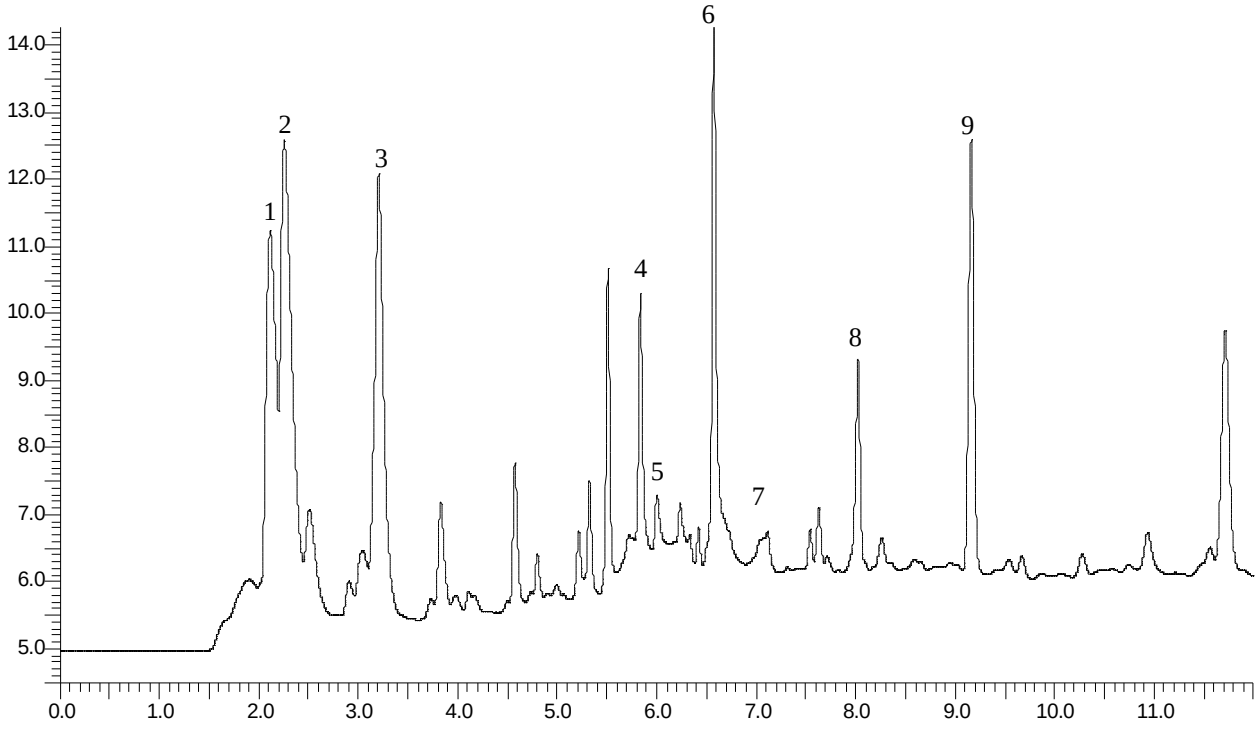

Figure 44. Stage 2 bananas + fermented cells 48 hrs

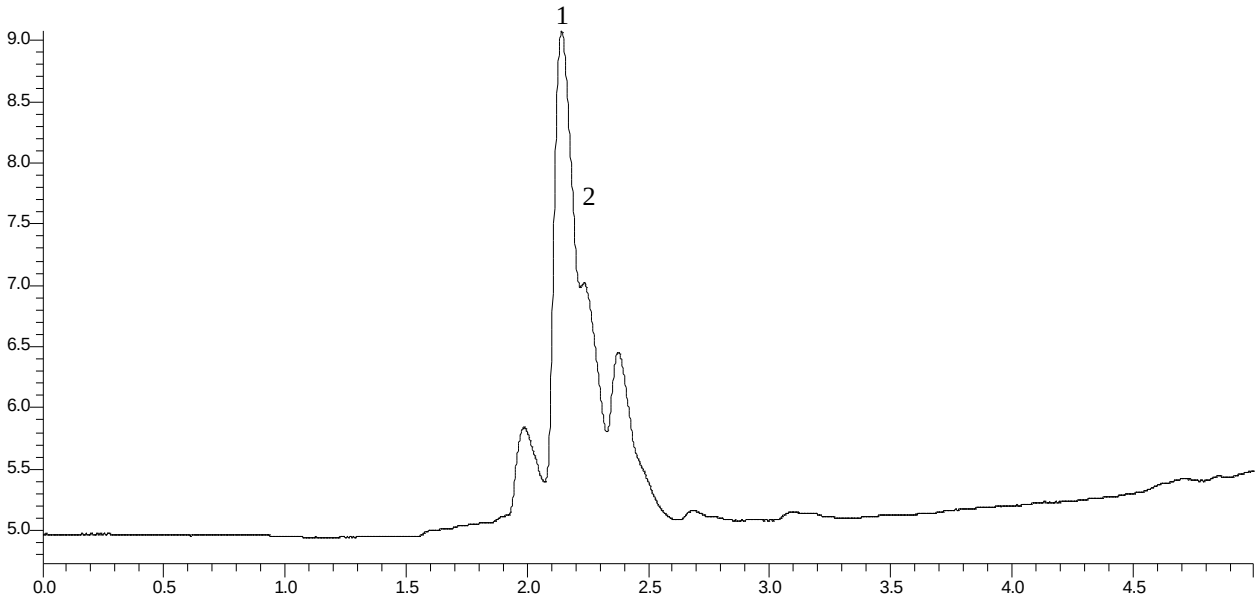

Figure 45. Fermented cells 48 hrs

3.3.10 Volatiles Production from Fruit and Rhodococcus (GC Method 3)

Compared to direct headspace injection, SPME fiber headspace injection showed different peaks and area counts among different *R. rhodochrous* DAP 96253 catalysts (Table 18). *R. rhodochrous* DAP 96253 catalysts, either live cells scraped from YEMEA plates with CoU, U, or uninduced, or GA-PEI immobilized catalysts (cells from fermentation with urea as the inducer), showed peak 1 and 2 (Fig 46-49). The area counts of peak 1 for *R. rhodochrous* DAP 96253 cells from YEMEA with cobalt and urea (CoU) and uninduced was much smaller than cells from YEMEA with urea (U) and GA-PEI immobilized catalysts. GA-PEI immobilized catalysts had the largest area count of peak 2, still, *R. rhodochrous* DAP 96253 cells from YEMEA with urea (U) had larger area count of peak 2 than cells from YEMEA with cobalt and urea. At retention time about 4.6 minutes, there was a large peak 3, which was detected on cells from YEMEA with urea (U), while at retention time about 8.4 minutes, there were several large peaks, i.e. peak 4, which was detected on cells from YEMEA with cobalt and urea (CoU) (Table 18).

After 20 hours incubation at 30°C, and 1 hour SPME fiber incubation at RT, the absorbance of SPME fiber of bananas and bananas with various *R. rhodochrous* DAP 96253 cells were analyzed (Table 19; Fig 46-54). Control bananas showed more peaks and larger area counts than bananas with *R. rhodochrous* DAP 96253 (Fig 50-54). There were many volatile peaks between retention time 4 minutes and 8 minutes for control analysis, whereas only a few peaks with comparable small area counts detected for bananas with *R. rhodochrous* DAP 96253. Effects on bananas ripening based on volatile production of live catalysts, *R. rhodochrous* DAP 96253 cells from YEMEA plates with cobalt and urea (CoU), urea (U), or without any inducers (uninduced) were better than glutaraldehyde immobilized *R. rhodochrous* DAP 96253 (prepared from fermented *R. rhodochrous* DAP 96253 with urea as the only inducer). Compared to *R. rhodochrous* DAP 96253 cells grown on different YEMEA plates, bananas with cells scraped from YEMEA plates with cobalt and urea as inducers (CoU) had fewer peaks and smaller area counts of all peaks except for peak 6 shown in Table 18 than cells from YEMEA plates with urea as the only inducer and uninduced YEMEA plates. For peak 1, only bananas with *R. rhodochrous* DAP 96253 from CoU plates and immobilized *R. rhodochrous* DAP 96253 showed smaller area count than control (Table 19).

Table 18. Peaks and area counts of peaks of *R. rhodochrous* DAP 96253 catalysts (SPME fiber headspace)

Peak	1	2	3	4
Retention Time (min)	~2.8	~3.4	~4.6	~8.4
R. rhodochrous DAP 96253 (GP)	90%	100%	~0	~0
R. rhodochrous DAP 96253 (uninduced)	1%	1%	~0	~0
R. rhodochrous DAP 96253 (U)	100%	28%	100%	~0
R. rhodochrous DAP 96253 (CoU)	19%	12%	~0	100%
Relative 100%	389705.08	19865.26	346648.50	31596.09

CoU: YEMEA with cobalt and urea; U: YEMEA with urea; GA-PEI: glutaraldehyde immobilized *R. rhodochrous* DAP 96253 (cells from fermentation with urea as the only inducer)

Table 19. Peaks and area counts of peaks of bananas with various *R. rhodochrous* DAP 96253 cells (SPME fiber headspace)

Peak	1	2	3	4	5	6	7	8
Retention time (min)	~2.6	~3.4	~4.1	~4.2	~5.4	~5.7	~7.5	~8.1
BA 1	100%	100%	100%	*	100%	100%	100%	100%
BA 1 + *R. rhodochrous* DAP 96253 (CoU)	13%	10%	5%	76496.57	5%	104%	~0	5%
BA 1 + *R. rhodochrous* DAP 96253 (U)	105%	20%	15%	338702.86	~0	15%	1%	4%
BA 1 + *R. rhodochrous* DAP 96253 (uninduced)	112%	19%	23%	204519.75	~0	14%	1%	10%
BA 1 + *R. rhodochrous* DAP 96253 (GA-PEI)	70%	42%	*	*	108%	*	10%	31%
Rela-tive100%	369867.77	320727.23	251711.69	25083.93	201536.16	180376.19	1320791.93	178509.36

BA 1: bananas in stage 1; CoU: YEMEA with cobalt and urea; U: YEMEA with urea; GA-PEI: glutaraldehyde immobilized *R. rhodochrous* DAP 96253 (cells from fermentation with urea as the only inducer)

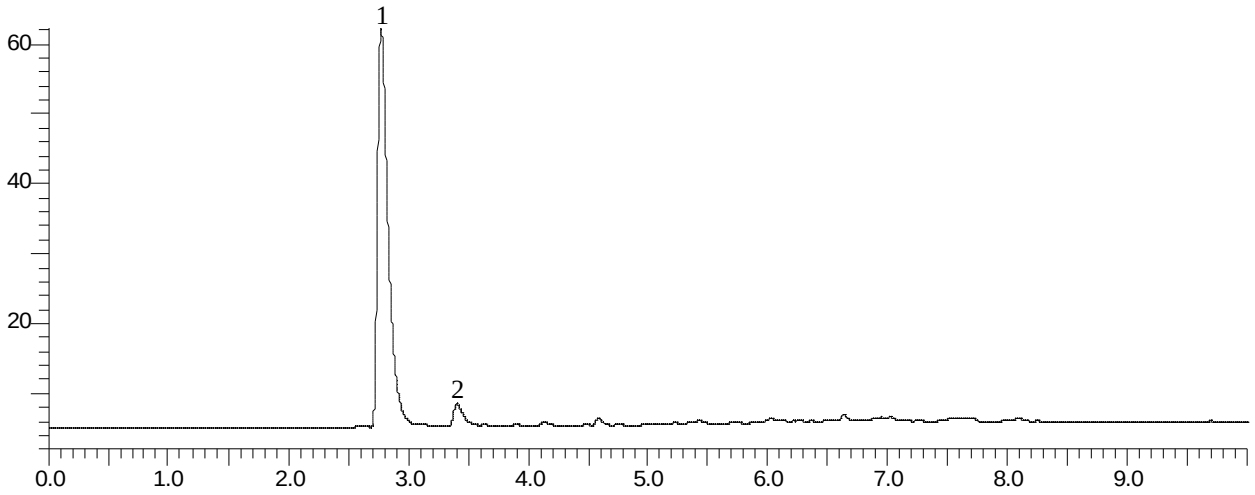

Figure 46. GA-PEI *R. rhodochrous* DAP 96253 from bioreactor with urea

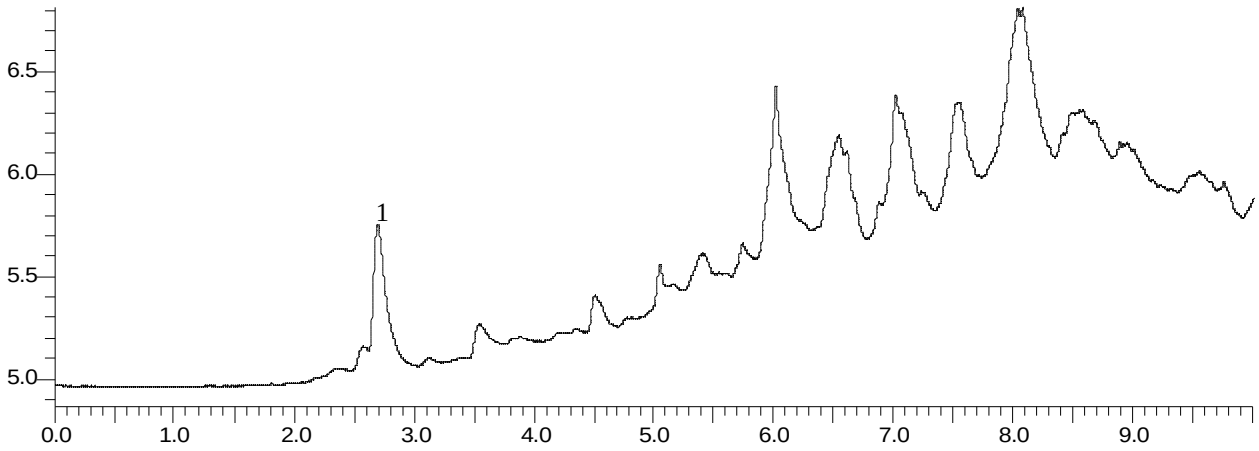

Figure 47. *R. rhodochrous* DAP 96253 (uninduced)

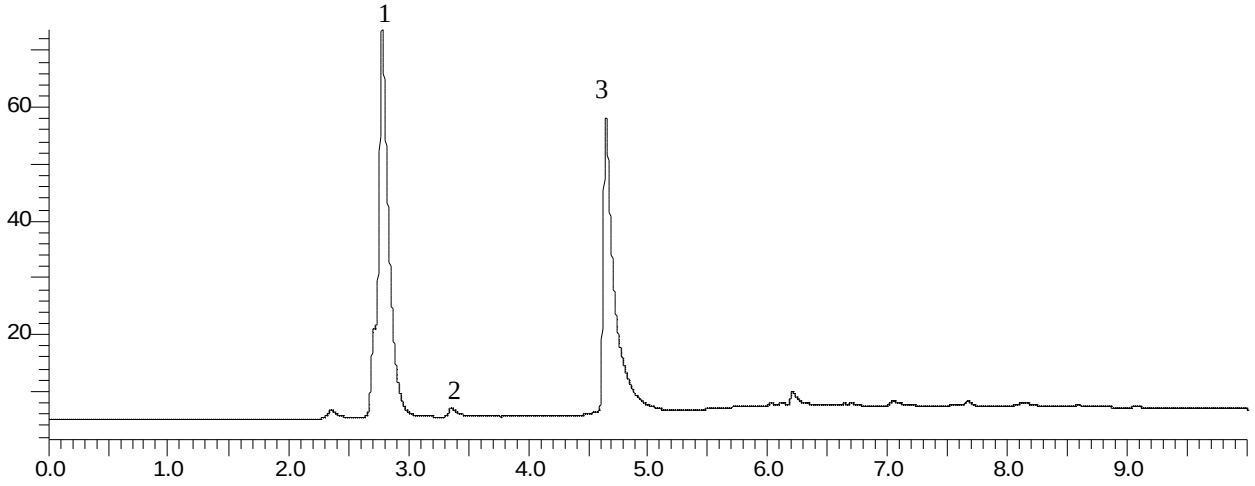

Figure 48. *R. rhodochrous* DAP 96253 (GU)

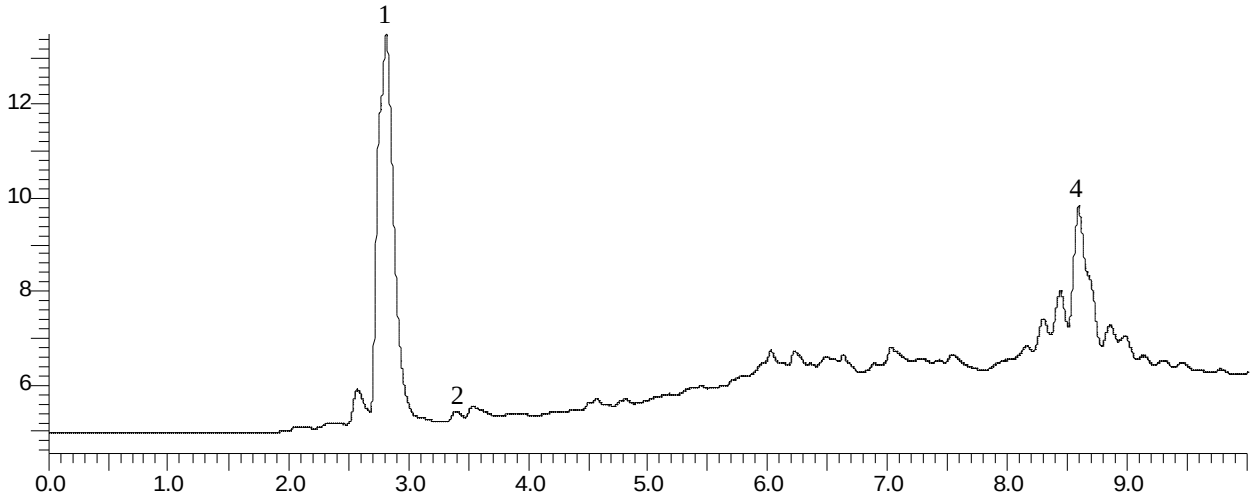

Figure 49. *R. rhodochrous* DAP 96253 (CoU)

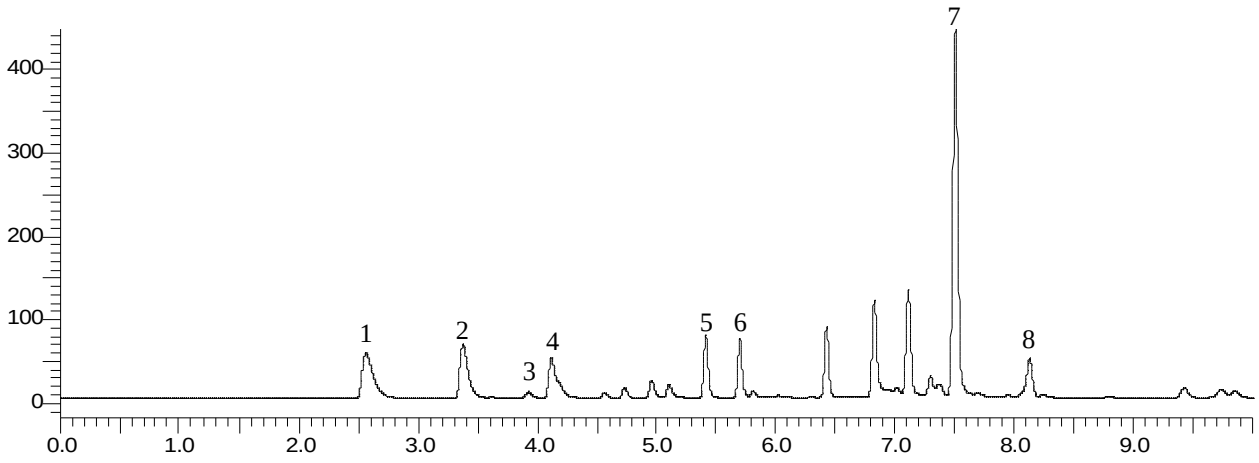

Figure 50. Bananas (Stage 1)

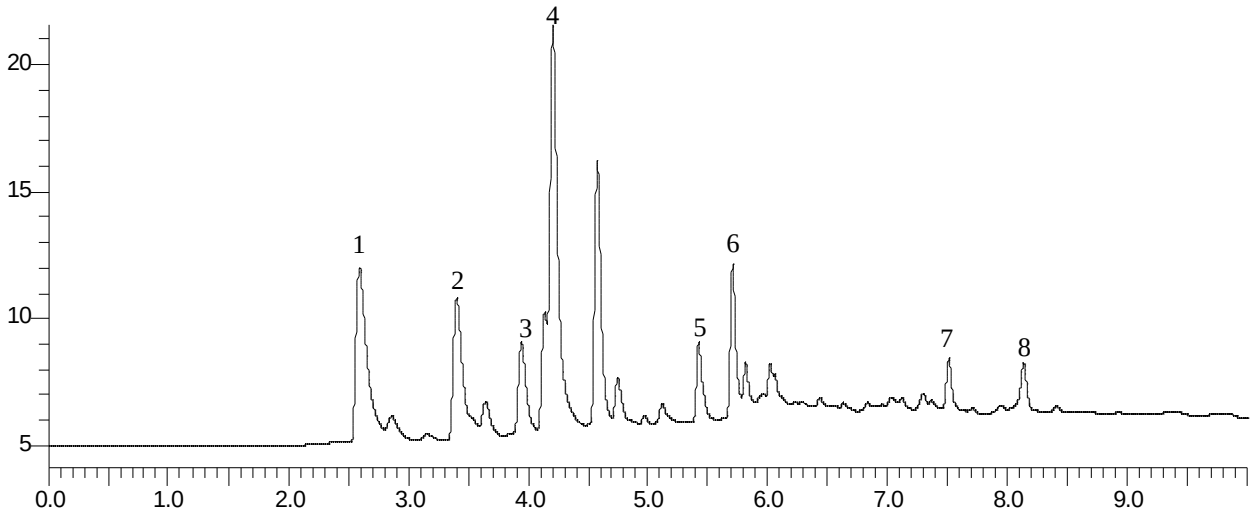

Figure 51. *R. rhodochrous* DAP 96253 (CoU) + Bananas (Stage 1) 7

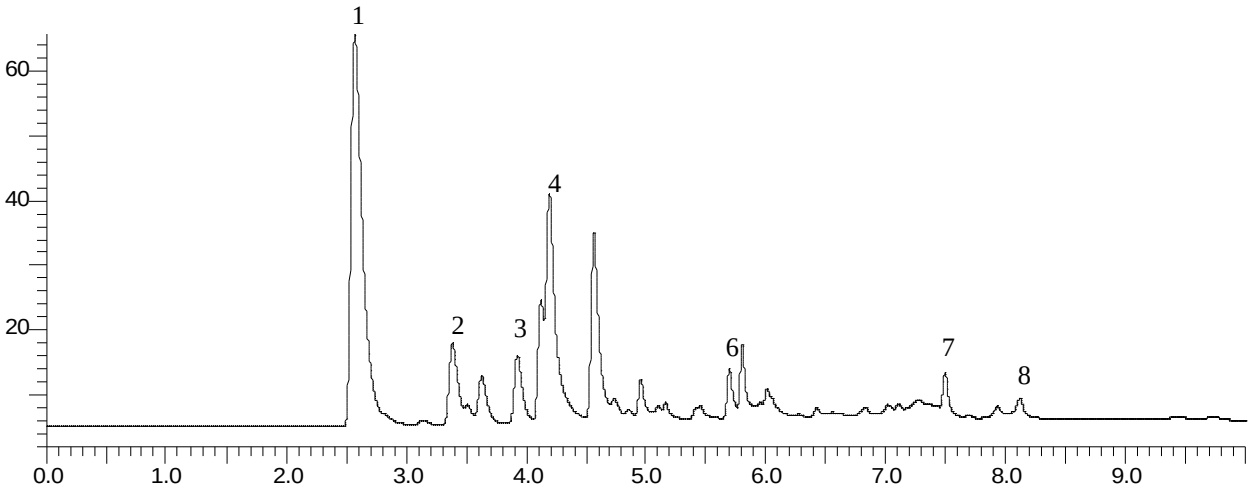

Figure 52. *R. rhodochrous* DAP 96253 (uninduced) + Bananas (Stage 1)

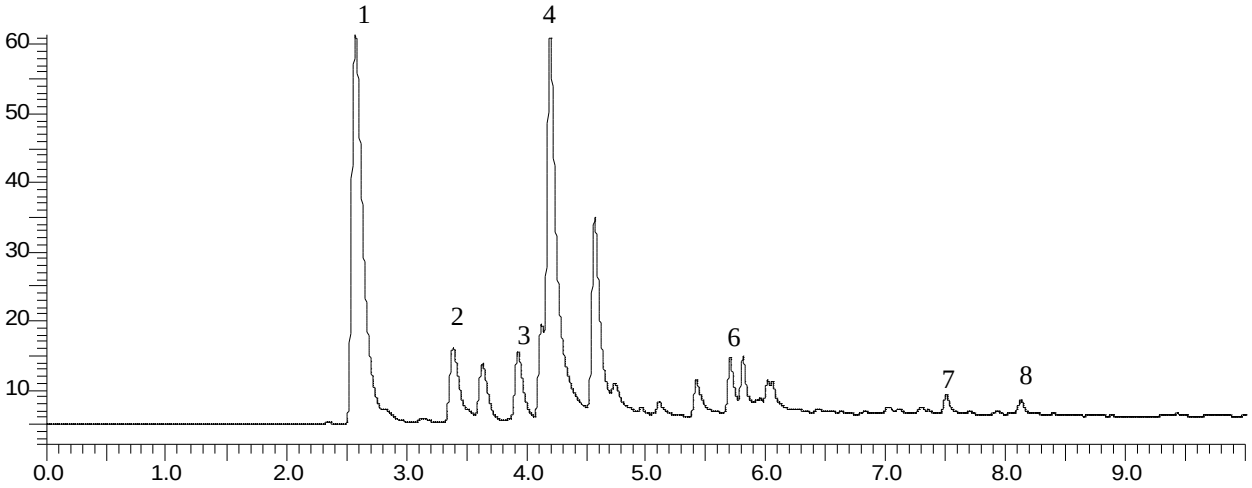

Figure 53. *R. rhodochrous* DAP 96253 (GU) + Bananas

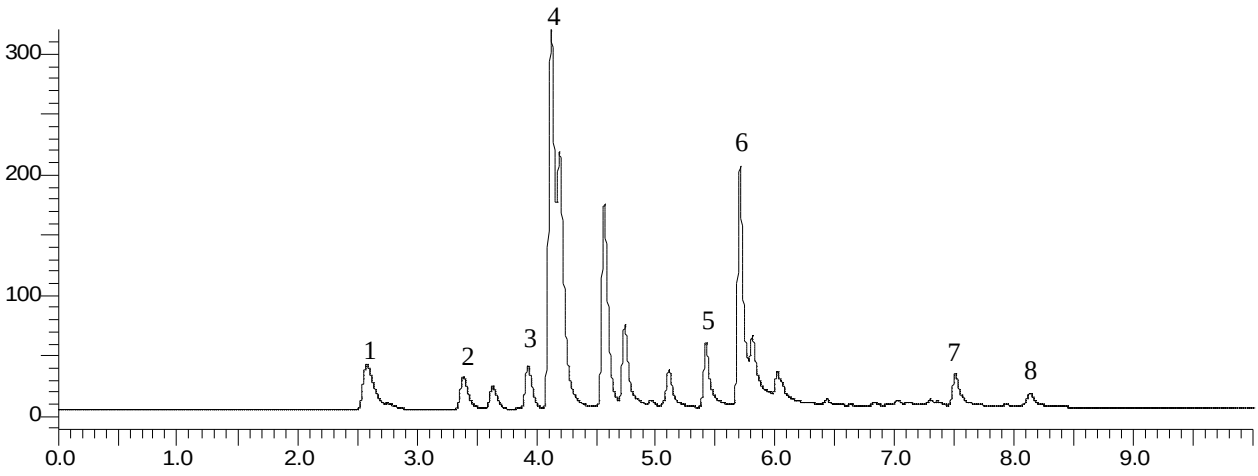

Figure 54. GA-PEI *R. rhodochrous* DAP 96253 from bioreactor with urea + Banana

3.4 Fungal Inhibition of GP Immobilized Catalyst

After 48 hours, antifungal activity of the catalyst was observed as *Penicillium spp.* grown on 10% SAB showed less sporulation (pigment) than the control (Fig 55-A), and *A. niger* grown on uninduced YEMEA showed less sporulation (pigment) than control (Fig 56-B), while on 10% SAB, there was no significant difference compared with the control (Fig 56-A).

After 72 hours, the fungal inhibition was more significant, especially for *Penicillium spp.* shown on 10% SAB (Fig 57-B).

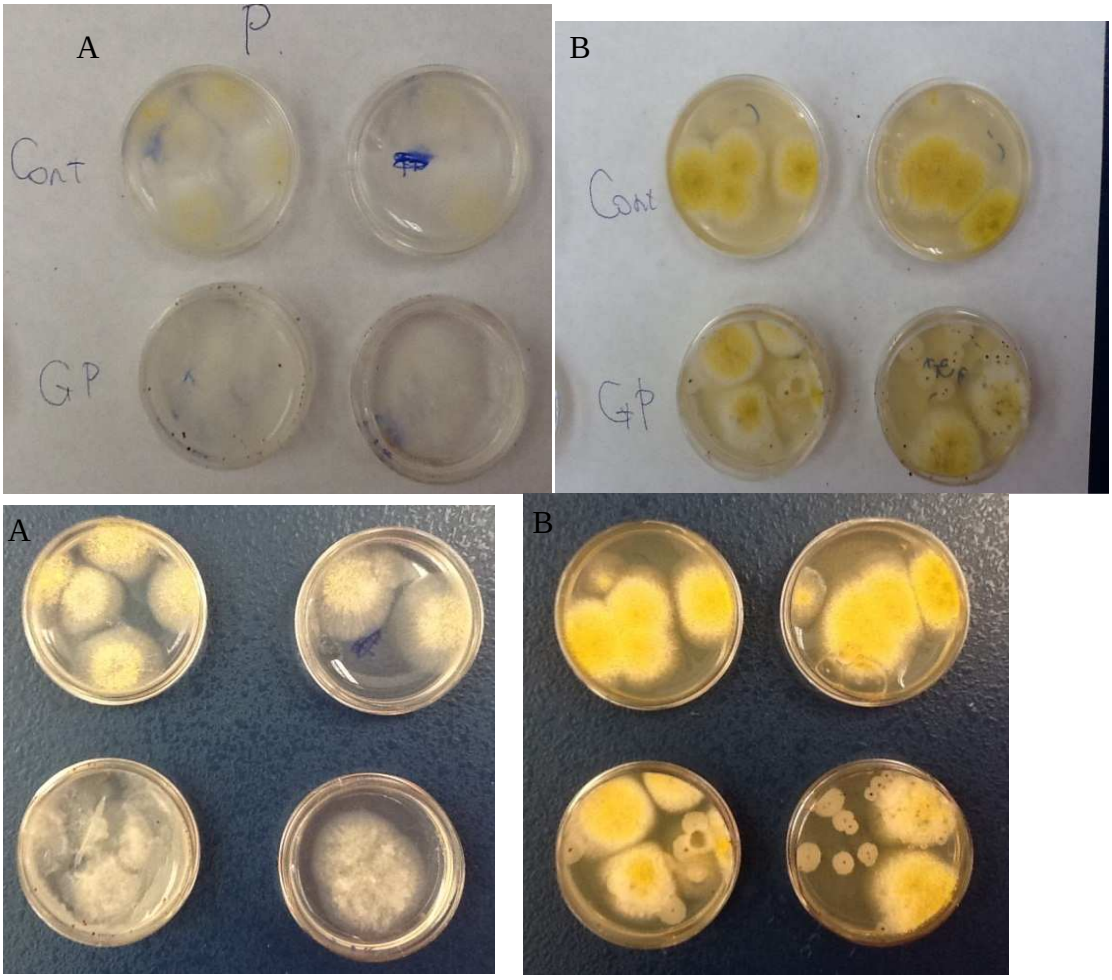

Figure 55. Effects of GA-PEI catalyst on growth and spore germination of *Penicillium spp.* on uninduced YEMEA (A) and 10% SAB (B) after 48 hours incubated at 30°C

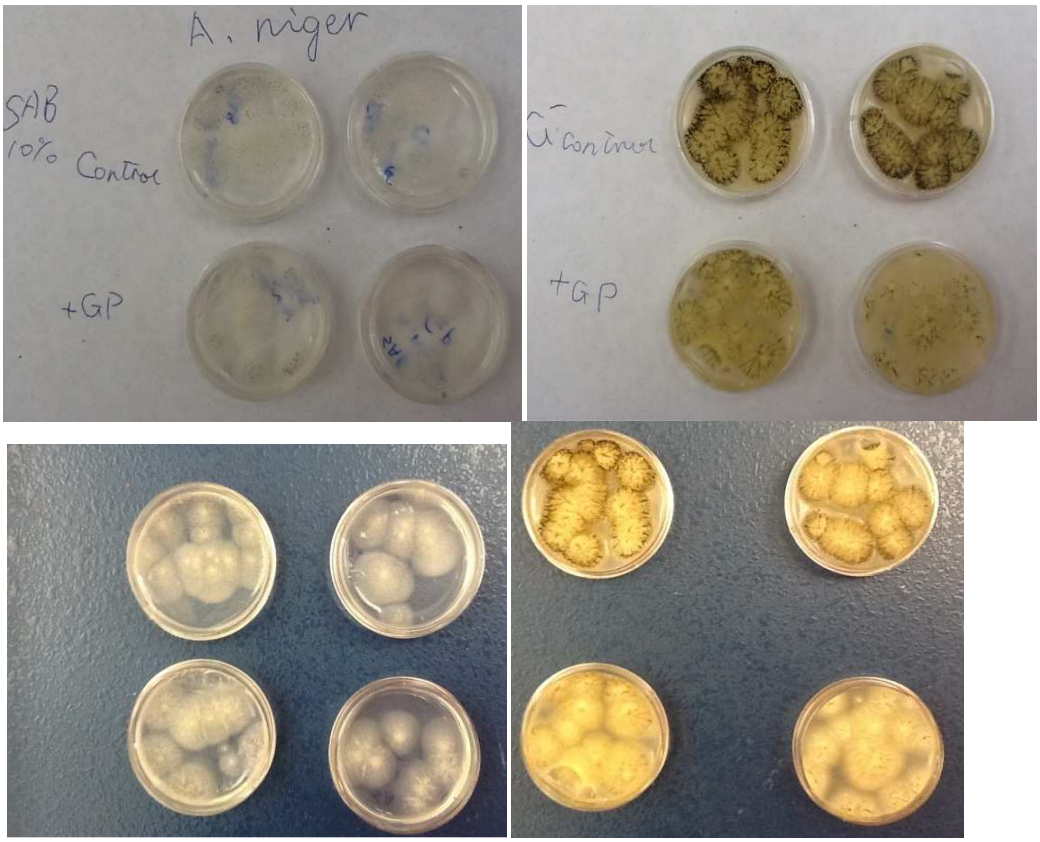

Figure 56. Effects of GA-PEI catalyst on growth and spore germination of *A. niger* on uninduced YEMEA (A) and 10% SAB (B) after 48 hours incubated at 30°C

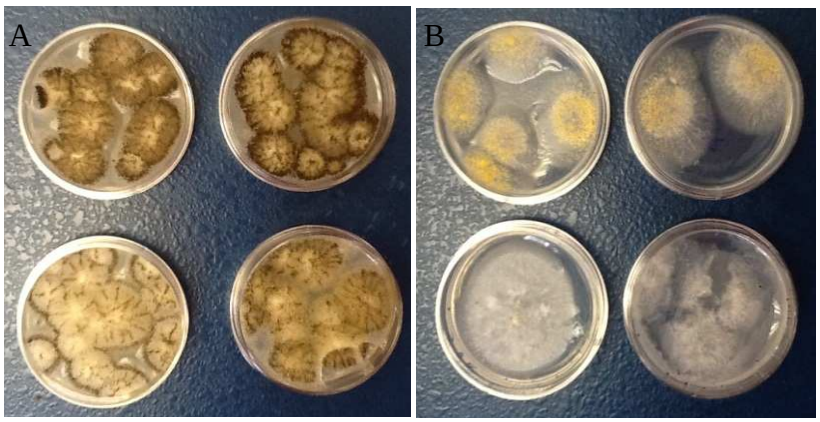

Figure 57. Effects of GA-PEI catalyst on growth and spore germination of *A. niger* on uninduced YEMEA (A) and *Penicillium spp.* on 10% SAB (B) after 72 hours incubated at 30°C

4 DISCUSSION

Inducing activity of key enzymes through the modification of the medium could lead to an improved catalyst that can be used in delaying the fruit ripening process. The effects of modified media on the induction of enzymes involved in fruit ripening have been examined in this study. There was no significant difference among various inducers tested in this study on βCAS-like enzyme production. Inducers such as cobalt and iron, which could be considered as the metal in active site of NHase, combined with urea in the growth media significantly induced the NHase activity in *R. rhodochrous* DAP 96253. Urea, as an organic fertilizer, has been reported to increase the ACC deaminase production of the plant growth promoting rhizobacteria (Naveed *et al.*, 2008). In this study, it was shown that urea also induced induction on ACC deaminase, amidase, and cyanidase. Meanwhile, the inorganic fertilizer, KNO3, was tested, which showed that it induced the three enzymes well but less effective than urea. When methacrylamide, a substrate for amidase, was added in the growth media of *R. rhodochrous* DAP 96253, all five enzymes were induced. Additional amino acid such as serine and cysteine in growth media, did not affect the enzyme production. However, glutamine and asparagine, whose transamination involved in amidase and NHase, showed enzyme induction ability on different enzymes, i.e. glutamine could induce amidase while asparagine could induce ACC deaminase. With toxic compounds such as KCN or Pb, there was no induction on enzyme productions except for βCAS-like enzyme.

Trehalose is a stable and biologically active disaccharide which consists of two subunits of glucose (Elbein *et al.*, 2003). In addition to serving as a simple carbon source in growth media, trehalose could be used to enhance the tolerance of organisms to hash conditions, i.e. heat, dehydration, and oxidative stress (Elbein *et al.*, 2003; Tucker *et al.*, 2012). It has been reported

that *Rhodococcus* could produce different types of trehalose containing glycolipids (Franzetti *et al.*, 2010). Sucrose is a disaccharide that consists of one glucose and one fructose units, which also is a broadly used sugar in daily life. Both sucrose and trehalose could be used as preservatives in industry. Maltodextrin is an oligosaccharide with multiple units of glucose. Maltose, fructose, and glucose are commonly applied reducing sugars that relate to sucrose and trehalose synthesis and metabolism, moreover, these three sugars can transform to each other under certain conditions, i.e. enzyme treatment. In this study, *Rhodococcus* could utilize all the six sugars tested. This study shows that changes of sugar content and amount affect enzyme activity. Trehalose significantly increased the production of NHase, amidase, and cyanidase, while maltodextrin increased the production of ACC deaminase and slightly βCAS-like enzyme. Decreasing the amount of sugars in media did not significantly affect the activity of amidase, cyanidase, ACC deaminase, and βCAS-like enzyme, whereas NHase activity increased while sugar amount increased. It was interesting that the mixture of sugars showed better enzyme induction than any single sugar tested, i.e. with a trehalose and sucrose mixture cells showed the highest NHase activity, 35.2% more than normal 4 g/L glucose, and even more than trehalose and sucrose only.

There are few studies that relate to stability of the enzymes present in induced cells of *R. rhodochrous* DAP 96253. This study concentrated on assessing and enhancing the stability of the five enzymes: NHase, amidase, ACC deaminase, cyanidase, and βCAS-like enzyme, produced in *R. rhodochrous* DAP 96253 by modifying the storage buffers and immobilization methods under different storage conditions. Because we know medium composition affects: 1) induction of these five enzymes, and 2) cell wall composition, media composition initially was investigated to determine if altering media components also affects enzyme stability in live cells

and in immobilized cells (non-replicating) catalysts. The *rhodococcal* cells were initially used as a whole cell catalyst. The use of the whole cell as a catalyst was cheaper than purifying the enzymes. Since the cells are always stored before application in fruit ripening and other processes, enhancement of stability of five enzymes are valuable. Previous work had shown that ACC deaminase, amidase, and NHase activities have different levels of loss after storage at -20˚C for 150 days. Hence, the development of proper storage buffers for the cells also is an important consideration.

PB appears to be fine for initial activities, but is less so upon long-term storage at various temperatures (Fig 23-26). This suggests for quick use of a catalyst, PB can be a storage buffer, however, for long-term storage, a buffer with supplements is needed. Sugars, such as trehalose and sucrose mentioned above, were used in preservation for bio-molecules in industry and research, i.e. it was reported that trehalose in storage buffer could enhance the stability of protein or purified enzyme when at room temperature (Elbein *et al.*, 2003). One objective of this research was to examine the relationship of whole cell catalysts and fruit ripening, since in real markets, the common temperatures for storing fruits are 4°C, 15°C, and room temperature, and during transportation, some tropical fruits may come across temperatures above 37°C. When applying catalysts to fruits, the key enzymes in whole cells need to be stable and show certain activity, hence, effects of sugars in storage buffers on stability of key enzyme activities were detected. After vacufuge drying at 30°C, *R. rhodochrous* DAP 96253 cells still could be considered as live catalysts (viability test). The cells dried with protection of different sugars showed different enzyme activity as well as cell suspension in sugar buffers. Among the six sugars tested in this study, trehalose, sucrose, and maltodextrin, captured most the attention. For cell suspension, both short time incubation and longtime storage, at lower temperatures, i.e.

4°C, maltodextrin in storage buffer showed the best protection on key enzyme activities, especially for NHase and amidase, while higher temperatures, i.e. 37°C and 55°C, trehalose and sucrose showed the best protection. For dried cells, sugars showed similar results as cell suspensions, however, maltodextrin especially retained most of NHase activity at all temperatures. The enzymes' activities of dried cells, i.e. NHase and Amidase were less stable than cell suspensions. At higher temperatures, i.e. 55°C, only cells dried under the protection of trehalose or sucrose showed some enzymes stability for all enzymes tested. Maltodextrin showed less stability than trehalose and sucrose for all the enzymes tested, but higher than the reducing sugars: glucose and fructose. βCAS-like enzyme, was comparably stable and no significant different among various sugars. Phosphate buffer, in certain conditions, showed strong ability to protect enzyme activity, i.e. short time storage (30 minutes) for ACC deaminase at various temperatures.

Immobilization catalysts were widely used in modern industry. *R. rhodochrous* DAP 96253 cells were immobilized with calcium alginate, glutaraldehyde, and wax and stability assessed. Among the three immobilization methods, calcium alginate immobilized cells were live catalysts in soft capsules stored with buffers, wax immobilized cells were live and dried catalysts, however, cells were not tightly immobilized since this method was not based on cross-linking, GA-PEI immobilized cells were non-replicable and dried catalysts. Comparing to lyophilized cells and PVA immobilized catalysts (commercially provided), wax and GA-PEI immobilized catalysts showed similar enzymes activities, calcium alginate and PVA cells were unstable for NHase and amidase, lyophilized cells could maintain most amidase and ACC deaminase. To control fruit ripening, a more effective catalyst for industrial applications is more applicable and

less energy consumptive than traditional methods. GA-PEI catalysts were chosen for further research since they could be easily reused as the dried particles without any storage buffers.

Slower ripening (or prolonged ripening) means longer shelf life and significantly reduces postharvest loss. Therefore, controlling fruit ripening was considered to be a major aspect to protect the quality of fruits. Although cobalt was reported as the ethylene receptor inhibitor in some papers, *R. rhodochrous* DAP 96253 from uninduced YEMEA or YEMEA with urea only still showed effects on delaying fruit ripening. This study showed that three stains of *Rhodococcus*: *R. rhodochrous* DAP 96253 and 96622 could delay bananas ripening to some extent, while more studies need to be done on *R. erythropolis* ATCC 47072. Among the three strains, *R. rhodochrous* DAP 96253 showed most significant effect on delaying fruit ripening. Based on the results of the induction and stability studies, *R. rhodochrous* DAP 96253 cells with the multiple inducers cobalt and urea that have highest enzymes activities and stabilities were used in fruit ripening experiments. It was interesting that cells were not only able to delay the ripening of ripe green bananas, but also could delay the ripening of ripe yellow bananas with golden-yellow color before the black spots appeared. Fermented *R. rhodochrous* DAP 96253 cells, which were with 48 hours growth time instead of 168 hours in plates, showed similar effects on delaying fruit ripening. Although the enzyme activity in immobilized cells was less than live cells, both immobilized and live *R. rhodochrous* DAP 96253 showed ability of delaying bananas and peaches ripening. Decreasing the amount of catalysts exposed to bananas caused less effectiveness on delaying fruit ripening. Spreading or dipping fruits with wax emulsion was a universal method that applied on peaches, apples, avocados, and etc., for protecting their qualities. Other than non-contact experiment, wax emulsions with *R. rhodochrous* DAP 96253 cells spreading on peaches were practiced, and peaches ripening were delayed in the cell-contact ex-

periment as well. And comparing with the control, the wax-cells coating peaches showed less fungi growth. Later studies on antifungal activity showed that immobilized *R. rhodochrous* DAP 96253 cells could delay the maturation of certain fungi to some extent.

Base on the results from GC, it was clear that cyanide (there was cyanide production during fruit ripening) could be degraded by either live cells or GP catalysts. Live *R. rhodochrous* DAP 96253 cells produced several peaks that may relate to fruit ripening, while GP catalysts have less volatiles produced but also affect volatile production during fruit ripening. Un-ripe bananas have more starch production, thicker peels, less volatile production than ripened bananas. Exposed with *R. rhodochrous* DAP 96253 could delay the appearance of the ethylene peak that happened as an initial of ripening in bananas. Further research will be more concentrated on defining the key volatiles associated with fruit ripening and how did they work on interfering with fruit ripening.

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